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Mechanism of Cell Division

I. Changes in Pyridine Nucleotide and DPNH-Cytochrome *c* Reductase in *Tetrahymena geleii W.* during the Course of Synchronous Culture*

(Dedicated to Prof. K. Kodama on the occasion of his 70th birthday)

By Tomoya Kamiya and Taijo Takahashi

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(Received for Publication, March 10, 1961)

Much work on the biochemical events taking place during the course of synchronous culture of *Tetrahymena* (*1*, *2*) has been accomplished (*3*). The principal feature of the culture lies in that synchronization was induced during the temperature treatment of 8 cycles between 34°C and 29°C, and during the time DNA,** RNA, and protein were observed to be synthesized (*3*, *4*). When cells were transferred to an optimal temperature, some mechanisms were assumed to begin to work and cells were found to prepare for synchronous divisions after 80 minutes. Some biochemical changes during this period seemed to be directry related to cell division cycle.

Preliminary experiments (*6*) showed that a change in the level of oxidized pyridine nucleotide occurred during this period in the synchronous culture of *Tetrahymena geleii W.* In this paper, change of the level of ox. PN, delay and inhibition of cell division caused by the addition of NA, and the effect of NA on the level of ox. PN were studied. To explain these findings the effect of NA and temperature change on the activity of DCR of *Terahymena in vitro* were examined. Furthermore the possible significance of DCR on cell division was presented and discussed.

MATERIALS AND METHODS

Experimental Organism and Culture Method—All of the experiments were carried out using *Tetrahymena geleii W* which was obtained through the courtesy of Dr. Ishii of the Hosei University. Cells were grown in a medium containing 2.0 g. of Polypeptone (Takeda), 0.5 g. of yeast extract (Takeda), and inorganic salt mixture, as described by Kidder and Dewey (*7*), in 100 ml. of distilled water. The basic procedure employed in the synchronous culture was the same as that described by Scherbaum and Zeuthen (*1*, *2*), and by Kamiya (*4*). The culture incubated at two different temperatures for 7.5 hours; during which the temperature was rhythmically changed between 28.5°C and 34°C at 30 minute intervals.

Analytical Methods—Determination of ox. PN was carried out as follows: 5 ml. aliquots of the cell suspension were taken from the culture flask at different stages of the synchronous culture, and rapidly filtered using Seitz filter apparatus through two filter papers (Toyo Filter Paper No. 7) and an asbestos filter disk from the top. Cells were retained on the top thin filter paper disk washed with 10 ml. of 0.5 % sodium chloride, and were rapidly extracted with ice-cold 10 % perchloric acids together with the filter paper. The time necessary for this procedure was less than 1 minute. After 10 minutes, the paper was disintegrated by a glass rod and centrifuged down. The ox. PN in the extract was determined by the fluorimetric method of Kaplan, using methyl

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* Major part of this work was presented at the 33rd Annual Meeting of the Japanese Biochemical Society, Tokyo, October, 1960.

** The following abbreviations are used in this paper: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; NA, nicotinamide; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide: TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; ox. PN, oxidized pyridine nucleotide; DCR, DPNH-cytochrome *c* reductase.

ethyl ketone reagent (*8*). The residue remaining after the extraction of acid-soluble compounds was dissolved in 0.2 *N* NaOH and protein content determined by the method of Lowry *et al.* (*9*). The protein value was corrected by subtracting that of the filter paper.

Oxidized and reduced forms of DPN and TPN were extracted according to the method of Glock and Mclean (*19*), and determined by the following enzymatic method: TPN and DPN by increase in the optical absorbancy at 340 mμ by glutathione reductase and alcohol dehydrogenase, respectively; TPNH and DPNH, by decrease in the optical absorbancy at 340 mμ by glucose-6-phosphate dehydrogenase and alcohol dehydrogenase (using acetaldehyde as the substrate), respectively (*10*). DPNH-cytochrome *c* reductase activity was determined by the method of Mahler (*11*).

Chemicals—The following pyridine derivatives were used; 6-aminonicotinamide kindly supplied by Frank W. Horner Ltd., Montreal; pyridine-3-sulfonic acid obtained through the courtesy of Dr. Mizuno of the National Institute of Health, Tokyo; nicotinamide methiodide, 2-anilinopyridine, nicotinic acid, 3-sulfopyridine 1-oxide, N-2-pyridylpyridinium perchlorate, and nicotinamide 1-oxide kindly provide by Dr. Hamana of this University. Nicotinamide used was the product of Ishizu Pharmaceutical Co., Ltd.,

RESULTS

Change in PN Content of Synchronized Tetrahymena—The contents of PN's in exponentially growing *Tetrahymena* are shown in Table I. DPN content was a little lower than DPNH and TPN was found mainly to be the reduced form. The level of oxidized TPN was negligible as was shown in the various tissues and organs (*19*). Fig. 1 represents the percentage change in the ox. PN content during the course of synchronized culture of *Tetrahymena*.

Table I

*Pyridine Nucleotide Content of Exponentially Growing Tetrahymena geleii W*1)

Coenzyme	μg./mg. of protein
DPN	1.65
DPNH	1.95
TPN	0
TPNH	1.70

1) Pyridine nucleotide content was determined on 0.1 *N* HCl and NaOH extract of *Tetrahymena* cells.

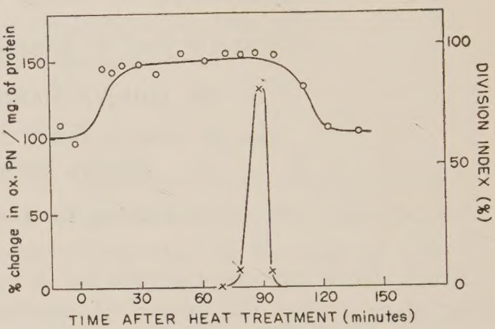

Fig. 1. Correlation between oxidized pyridine nucleotide content and cell division in *Tetrahymena*. —○—, ox. PN content; —×—, division index which was denoted as percentage of number of dividing cells per total cells.

It is thus apparent that the completion of heat treatments, as described previously, resulted in the rapid rise in the level of the ox. PN content of the acid-soluble fraction of the cells. The value at the later stage of the heat treatment was found to be equal to that of unsynchronized cells. This level increased by 30–40 per cent during the next 30 minutes after the heat treatment, ceased

Table II

Effects of Concentration and Time of Addition of Nicotinamide on Cell Division of Tetrahymena

Time of nicotinamide addition (minute after the heat treatment)	Concentration of nicotinamide (*M*)	Inhibition of cell division
30	0.1	+
30	0.05	+
30	0.01	−
30	0.005	−
0	0.05	+
30	0.05	+
60	0.05	+
90	0.05	−

+, synchronized cell division was completely suppressed; −, not supressed.

and attained a plateau which continued until the period of cell division, and then declined to the original level after 30 minutes following the synchronous division. This phenomenon was not observed when cells were collected by centrifugation or when heat-treated cells were transferred to an inorganic medium to result in the synchronized division as described by Hamburger and Zeuthen (*5*).

Inhibition of Cell Division and Increase of PN by NA—The effective concentration of NA on the inhibition of synchronized cell division is illustrated in Table II.

Cell division was completely inhibited in the presence of NA at a concentration of 0.05 *M* when added 30 minutes after the completion of heat treatment, while no effect was observed when added after 60 or 90 minutes. The effect of short exposure of synchronized cells to NA on cell division is shown in Fig. 2. Cells which were treated by the standard method of synchronization in an inorganic medium were exposed to 0.05 *M* NA (pH 7.0) at various stages of cellular growth cycle. Cells were allowed to contact with this agent for a period of 15 minutes and were transferred to the inorganic medium after two washings by mild centrifugations. Such exposure to NA resulted in the delay of cell division. Fig. 2 shows such delay as a function of the time when the cells were exposed to the agent. During the first 30 minutes, there occurred a sharp increase followed by a gradual decrease and then by a slightly sensitive period for about 20 minutes before the formation of the furrow in the cell. The time when the sensitivity reached the maximal level coincided with the time when the level of ox. PN attainted a maximum.

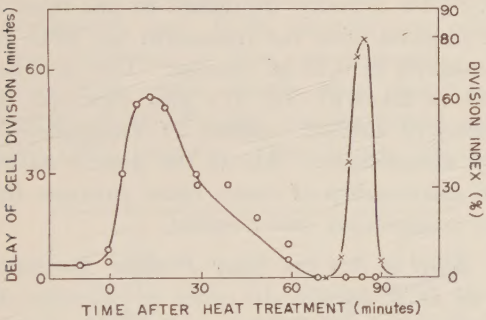

FIG. 2. Effect of short exposure of synchronized cells to NA on cell division.

—○—, effect of 0.05 *M* nicotinamide ;—×—, division index.

These results urged observation of the effect of NA on the ox. PN level. The change in PN level caused by the addition of 0.05 *M* NA was examined at three time intervals as indicated by the arrows in Fig. 3. When NA was added at 0 minute after the heat treatment, no increase of the PN level was observed. When added after 30 and 60 minutes, PN level alreaey attained a maxium (see Fig. 1) and gradually returned to the original value during the following 30 minutes after exposure to the agent. These observations suggested close relationship between the cell division and ox. PN level. The ox. PN may be considered to comprise mainly of DPN (see Table I).

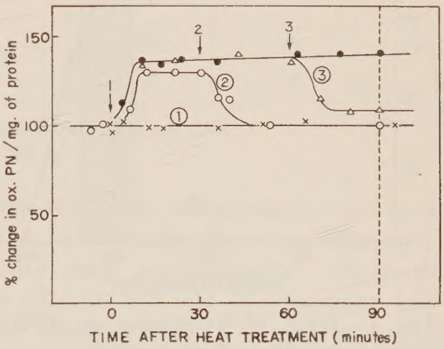

FIG. 3. Effect of addition of 0.05 *M* nicotinamide on the pyridine nucreotide content in synchronized cells of *Tetrahymena*.

Nicotinamide was added at three time intervals as indicated by arrows 1, 2, and 3. The change in oxidized pyridine nucleotide content was protted by curves 1 (—×—), 2 (—○—), and 3 (—△—) respectively. Dotted line shows time of maximum division index in control run (—●—).

Effect of Temperature on the Activity of DPNH-Cytochrome c Reductase in vitro—From the result of preceding experiments, it was concidered to be of interest to know what enzyme is responsible for the increase in the ox. PN level after the heat treatment (Fig. 1) and the delay of cell division caused by the addition of NA (Fig. 2). Among the many enzymes supposed to fit in the explanation of the observed phenomena, DCR was chosen as an enzyme system and nature of DCR concerning heat stability and sensitivity to various inhibitor were examined.

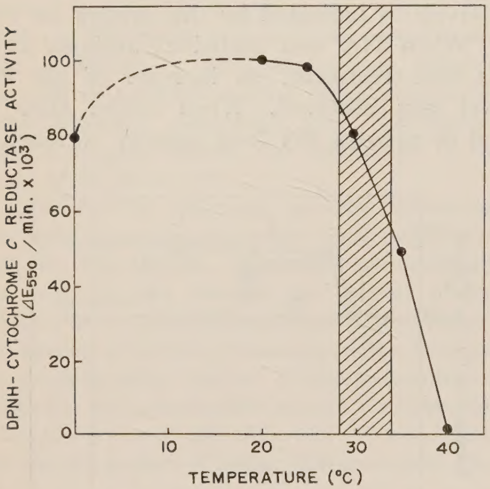

FIG. 4. Effect of temperature on DPNH-cytochrome *c* reductase of exponentially growing cells of *Tetrahymena*.

Supernatant (20,000×*g*) of disintegrated *Tetrahymena* cells was incubated in 0.25 *M* sucrose containing 0.025 *M* glycylglycine buffer (pH 8.5) for 10 minutes at various temperatures, and DPNH-cytochrome *c* reductase activities determined. Hatched area represents temperature range at which temperature cycling was applied on *Tetrahymena* cells.

The effect of temperature on DCR activity *in vitro* is shown in Fig. 4. The cells of log phase *Tetrahymena* were collected, washed, and homogenized with a conventional Potter-Elvehjem homogenizer in 0.25 *M* sucrose. The homogenate was subfractionated with the refrigerated centrifuge at 20,000 ×*g*. The supernatant thus obtained was treated for 10 minutes at various temperatures maintaining the pH 8.5 with the addition of 0.025 *M* glycylglycine buffer, and then chilled to 0°C. The DCR activity was then assayed on each supernatant thus treated. As shown in the figure, the activity declined linearly with the temperature increase from 30°C to 40°C and complete inactivation occurred at 40°C. The type of this inactivation seemed to be irreversible, since no recovery of the activity was found when the heat-treated samples were further incubated at 0°C. The interesting point is that the range of temperature which was applied to induce synchrony in cell division coincided with that which caused a partial loss of DCR activity. This range is indicated in the figure as a hatched area. Although it was shown in the present experiment (*in vitro*) that DCR was irreversibly inactivated by heat treatment, if DCR activity is inactivated reversibly by the heat treatment *in vivo*, the change in DCR activity during the heat treatment (28.5°C–34°C) would be responsible for the induction of synchrony. At pH 7.0, DCR activity was more stable to heat treatment than at pH 8.5. The activity decreased to one-third of the original after the treatment at 40°C for 20 minutes in 0.25 *M* sucrose. The previous work of Eichel (*16*, *17*) also reported the lability of DPNH oxidase of *Tetrahymena* to high temperature. About the precise nature and relationship of both these enzymes further exploration was awaited.

Effect of NA and Other Pyridine Derivatives on the DCR Activity—In order to examine the responsibility of DCR on the cell division further, NA and 9 other pyridine derivatives were tested for their inhibitory action on cellular activities relating to cell division. These agents were selected from 30 or more pyridine derivatives (unpublished) known to inhibit synchronized division of *Tetrahymena* when applied after the temperature cycling. The results are summarized in Table III, which shows the effect of these substances on the cell division, morphological characteristics, cellular motility, respiratory rate, and DCR activity. It was thus found that the motility, morphological characteristics and respiratory activity were affected only slightly by these agents at the level of 0.05 *M*,

TABLE III

Effects of Pyridine Derivatives on Various Cellular Activities

	Effect on motility1)2)	Effect on morphological characteristics1)2)	Inhibitory activity on cell division1)2)	Respiratory rate, per cent of control 2)	DCR activity, per cent of control	
					0. 05M	0.01M
Nicotinamide	—	—	+	80	70	—
6-Aminonicotinamide	—	—	+	94	69	87
Nicotinamide Methiodide	—	—	+	97	78	100
Pyridine-3-sulfonic acid	—	—	+	100	40	64
2-Anilinopyridine	—	—	+	104	73	98
Nicotinic acids	—	—	+	91	61	100
3-Sulfopyridine 1-oxide	—	—	+	97	74	101
N-2-pyridylpyridinium perchlorate	—	—	+	93	72	97
Nicotinamide 1-oxide	—	—	+	116	72	—

1) +, postive effect; —, negative effect. For further explanations see text.

2) all inhibitors were added at the concentration of 0. 05 M.

while the synchronized division was completely suppressed, and DCR activity was more or less inhibited at this concentration. It appears from these results that almost all inhibitors of cell division so far tested also inhibit DCR activity. Thus, it may be concluded that these substances have a common feature in that they have no effect on the motility and morphological characteristic, little effet on the rate of respiration and are rather inhibitory on DCR activity, especially on cell division at a relatively high concentration (0. 05 M).

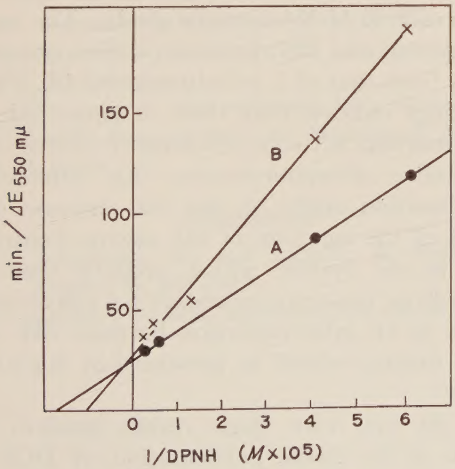

FIG. 5. Inhibition of nicotinamide on the activity of DPNH-cytochrome *c* reductase *in vitro* at pH 8. 5 (30°C)

Lineweaver-Burk plot in the absence (curve A) and presence (curve B) of 0. 05 M nicotinamide.

Kinetic analysis of DCR activity on 20, 000×g supernatant of *Tetrahymena* showed that the type of inhibition of DCR activity by NA was competitive. Fig. 6 indicats Lineweaver-Burk plot of the relative rate of the reduction of cytochrome *c* against different concentrations of DPNH in the presence and absence of 0. 05 M NA. The Michaelis constant of this enzyme reaction determined from the intercept of curve A on the abscissa was 5×10^{-6} M at pH 8. 5 (30°C).

DISCUSSION

In the present work, increase in the level of ox. PN, probably DPN, was considered to be related to cell division and DCR activity was also suspected to be responsible for the increase of ox. PN and for the cell division. These considerations are based on the following observations: (a) incubation of cells at 34°C, which inhibited cell division, also inhibited increase in the levels of ox. PN and DCR activity *in vitro*; (b) both of these activities were reversibly inhibited by 0.05 *M* of NA which was found to inhibit cell division at this concentration; (c) the first 30 minutes after the return to an optimal temperature is the time during which the amount of ox. PN increased, and the sensitivity of the cells for the inhibitory activity of NA on the cell division reached a maximal value; and (d) all of the pyridine derivatives tested were the inhibitors of cell division and of DCR activity. It is possible to infer from the present results, that DCR is the site of inhibition by heat treatment applied to induce synchrony in *Tetrahymena*.

The change in the level of ox. PN could not be demonstrated under such conditions that the medium was composed of inorganic substances (*5*) and that the cells were collected by centrifugation. It is conceivable that DPN-DPNH ratio in the cell is quite susceptible to environmental conditions, especially when an anaerobic state is brought about. Packing of cells by centrifugation and suspending them in an inorganic medium, indeed, were found to result in the decrease in respiratory activity to one-third the normal value. Confirmation of the precise nature of the increase of ox. PN must await further examinations.

Experiments on the short exposure of synchronized cells to NA suggest that some NA-sensitive system became to be active in the first 30 minutes after the end of heat treatment, resulting in the induction of cell division. Hamburger and Zeuthen found that during the first 50 minutes after the heat treatment, there was a gradual increase in sensitivity to 2,4-dinitrophenol as expressed by the delay of cell division (*5*). The difference between the sensitivity curve to NA and that to 2,4-dinitrophenol suggest that the NA-sensitive system is independent of the oxidative phosphorylation, or is indirectly related to the energy-yielding system. NA is known as a precursor of DPN in the liver of mammals which is transferred to the ribosyl group of phosphoribosyl pyrophosphate by DPN-pyrophosphorylase in the cell nucleus (*12*). NA has also been found to be a potent inhibitor of animal tissue DPNase (*20*). It shoud be noted that if NA acts as a precursor of DPN or inhibitor of DPNase, the resultant chanse in the content of DPN in *Tetrahymena* will be toward the increase of ox. PN after the exposure to NA. The present deta showed that the exposure of the synchronized cells to NA inhibited the rise of the level of ox. PN, presumably DPN. This suggests that the NA-sensitive system under consideration is neither DPN-pyrophosphorylase nor DPNase.

On the role of DCR in the cell division, there are two explanations. The one hypothesis is that DCR is connected to a system which provides the energy for cell division. Several informations about the accumulation of ATP and other nucleoside triphosphates (*13–15*) before the stage of cell division have been presented. Scherbaum discussed on the relationship between the energy system and DPNH oxidase activity (*3*) based on the information of heat lability of DPNH oxidase (*16*, *17*). Kamiya *et al.* found by a tracer technique that increase of ATP prior to synchronous division was inhibited by the addition of 0.05 *M* NA (unpublished). The sensitivity curve of NA, however, differs considerably from that of 2, 4-dinitrophenol (*5*). These findings indicate that there is some indirect relationship between NA-sensitive system and oxidative phosphorylation. An alternative explanation might be that this enzyme or a part of the function of the enzyme contributes to the system which prepares some organells or structures necessary for cell division, such as an interconversion between SH- and S–S during mitosis as presented by Rapkin (*21*).

At any rate these results confirm the view of the direct participation of DCR to the trigger mechanism (*18*) which has been assumed to initiate the synchronous division in this organism.

SUMMARY

Changes in oxidized pyridine nucleotide, nicotinamide sensitivity of cell division, and DPNH-cytochrome *c* reductase activity were studied in synchronously dividing cultures of *Tetrahymena geleii W* using enzymatic and chemical methods. In addition, the effects of temperature, nicotinamide and other pyridine derivatives were observed on these parameters.

The level of ox. PN increased after the heat treatment and decreased after cell division. This increase returned to the original value after exposure to 0.05 *M* NA. This concentration of NA inhibited completely the synchronous division of *Tetrahymena*. NA sensitivity curve showed its maximum within the first 30 minutes after the heat treatment. DPNH-cytochrome reductase activity was irreversibly inhibited by the heat treatment and competitively inhibited by NA *in vitro*.

The results of these and other experiments indicate that there may be a direct relationship among various phenomena, such as increase of ox. PN, change in NA sensitivity of cell division, DCR activity and cell division in synchronized cells of *Tetrahymena*.

We wish to thank Miss S. Kikuchi for her technical assistance in a certain phase of the present work and Mr. Sonoda for his help in the enzymatic determinations.

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Studies on the Gamma Ray Irradiation on the Protein

II. The ESR Absorption of Proteins, Amino Acids and Peptides

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(Received for publication, February 16, 1961)

As pointed out by Ingram (*1*), the electron spin resonance (ESR) absorption spectroscopy is one of the most direct methods for studying the free radical formation in the irradiated high polymer and seems very important to basic physical knowledge of breakdown process in the living system produced by irradiation. Indeed, some studies along such an approach to radiation injury of biologically important substances have already appeared (*2–4*). The present author has also reported preliminary results on the ESR spectra of γ-ray irradiated serum albumin (*5*) as well as some amino acids and peptides (*6*). As a continuation of the previous reports, this paper is chiefly concerned with observations on the ESR absorption spectra of γ-ray irradiated proteins.

METHODS AND MATERIALS

Samples Tested—Crystalline bovine serum albumin was Armour's product, while human serum albumin was prepared and crystallized by Cohn's method (*7*). Other compounds used were commercial ones, mainly obtained from Ajinomoto Co.

Irradiation—The procedure has already been described in detail in the previous reports (*5*, *6*). The 2 kilo-curie Co^{60} gamma-ray source at the Institute for Chemical Research, Kyoto University, was used, its intensity being 3×10^3 r per minute. Irradiation was usually made under vacuum (10^{-3} mm Hg), except that some samples were irradiated in the presence of air, oxygen, nitrogen or water to observe their effect on free radicals formed.

ESR Absorption—For weak signals, background signal originated from free radicals formed in glass of irradiated sample tube should be taken into account. Hence the sealed glass tube was opened, the sample was transfered into another unirradiated tube and then ESR signal was observed as quickly as possible. No appreciable oxygen effect was observed in our all samples tested immediately after exposure, though considerable change in the ESR signal was observed in some cases after several hours. When the signal was sufficiently strong, however, opening of the sample tube could be avoided by heating one end of the tube to reduce undesirable background signal as far as possible, to which a sample in the other end of tube was transfered carefully after cooling.

ESR spectra was observed by a hand-made ESR apparatus in earlier observations as in the previous experiments (*5*). In the later observations, however, Varian V-4500 spectrometer at the Resource Research Institute, Tokyo, was chiefly used because of its stability. The results presented here are principally provided by the latter, the tracing being the first derivative of the absorption curve.

RESULTS

The Pattern of ESR Spectra—Using ESR absorption, free radical formation by irradiation was examined on bovine and human albumin, fibrin, fibroin and silk fibre itself. Cysteine and cystine content of these proteins are varied from several to nearly zero per cent, their order being given as the following sequence;

> bovine albumin, ≧ human albumin, > fibrin, > fibroin and silk fibre

The typical pattern of their ESR absorption was presented in Figs. 1–3, together with that of some amino acids and peptides.

Such a free radical formation as proved by these ESR signals was always observed for dose of 10^5–10^7r, the more the dose, the larger the signal. Under the fixed conditions for signal recording, therefore, the dose-signal

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A part of the results in this paper was presented at the Kinki District Meeting of Japan Physiological Society (September, 1960).

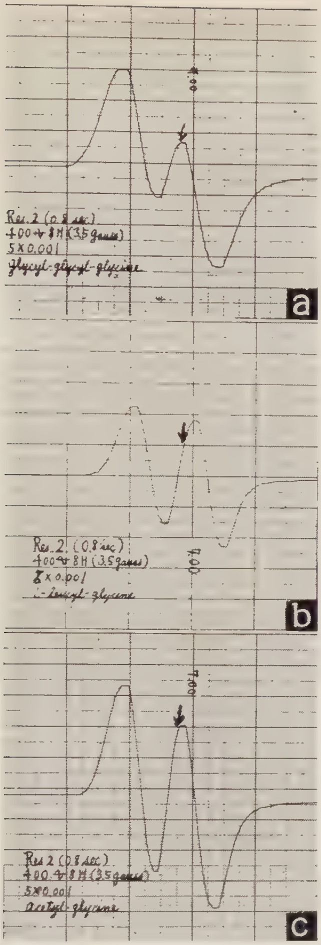

FIG. 1. ESR absorption spectra of γ-irradiated a) glycyl-glycyl-glycine, b) glycyl-leucine and c) acetyl-glycine in the absence of air.

relation was studied somewhat quantitatively on the bovine serum albumin. Irradiation with γ-rays less than 10^3 r caused hardly any noticeable ESR absorption, its signal being distinctly distinguished from background noises only when the dose of radiation exceeded 10^3 r. As illustrated in Fig. 4, an approximate linear relationship was obtained between the dose of γ-rays and the height of the highest component in the ESR signal for 10^3–10^6r at least. For the dose of 10^7r, the signal was too strong and sensitivity of the spectrometer had to be lowered and so its accurate comparison with these for lower doses was, strictly speaking, impossible. As to other proteins, circumstances appeared also similar. In the lack of systematic observations, however, something conclusive can hardly be said. On the other hand, lowering of spectrometer's sensitivity reduced troubles due to background noises and zero line drift, but seemed not to alter the character of ESR absorption. Thence the high dose of 10^7 r was adopted for comparing the characteristics of ESR signal of proteins and peptides as shown in Figs. 1–3.

It is obvious from the presented records that the ESR signal of bovine serum albumin shows close resemblance to that of cystine, cysteine and glutathione, while the pattern of fibroin and silk fibre resembles that of glycyl-glycyl-glycine or glycyl-leucine. The pattern of human serum albumin seems to be an intermediate type of the above two and that of fibrin rather similar to the latter. In other words, the more the protein contains sulphhydryl and disulphide groups, the more its ESR absorption resembles that of cysteine or cystine. Gordy and his coworkers (*8*) reported already that the ESR spectra of X-irradiated cystine and fibrous proteins rich in cystine (hair, feather) were seen to be very similar to each other. The present findings show good agreement with theirs. It seems worthy of note here that these spectra of cystine type are very similar to those obtained from frozen solution of polymeric sulphur in oleum (*9*). According to Ingram's observation made by various microwave frequencies, such a complex pattern of polymeric sulphur is not due to hyperfine structure but due to components of different g-values. When g-values of such components are estimated from the results obtained, *e.g.* from that of reduced glutathione, they are approximately 2.04, 2.027, 2.017 and 2.003 respectively (Fig 3B), and their agreement with those of polymeric sulphur radicals in dilute oleum is fairly well. Putting aside the question as to whether the mechanism proposed by Gordy *et al.* (*8*) is valid or not, it seems evident that radicals in the protein are associated with their cystine and cysteine content, and their breakdown process by irradiation

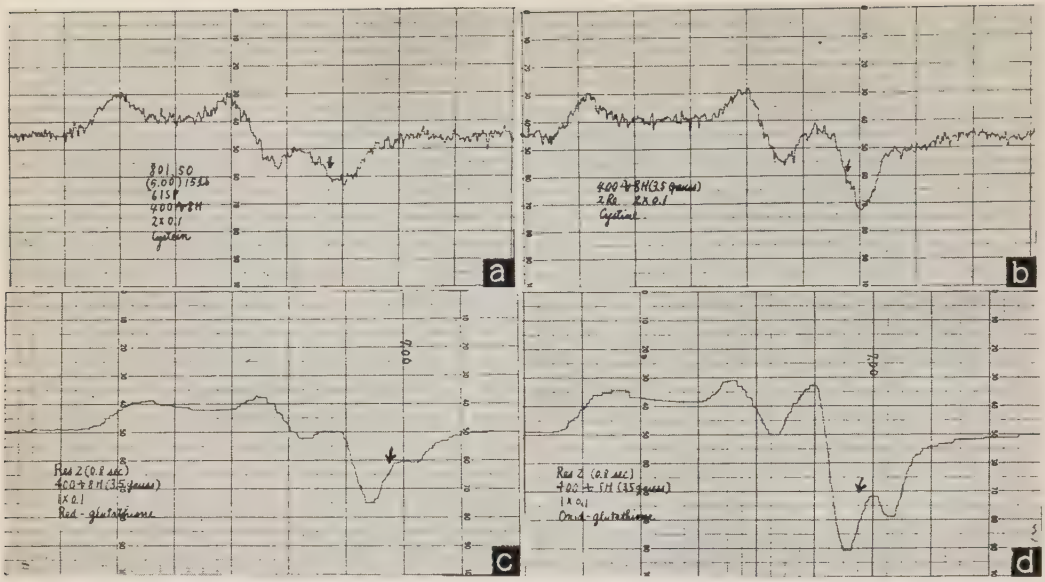

FIG. 2. ESR absorption spectra of γ-irradiated a) cysteine, b) cystine, c) reduced glutathione and d) oxidized glutathione in the absence of air.

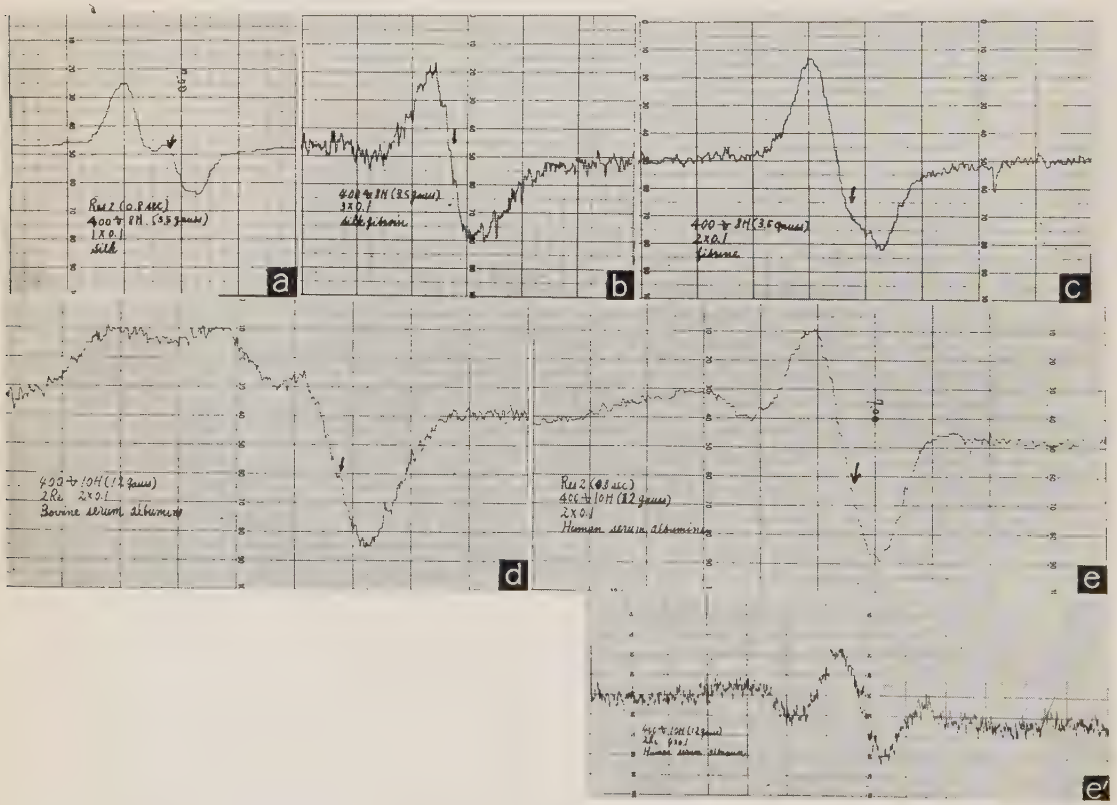

FIG. 3-A. ESR absorption spectra of γ-irradiated proteins in the absence of air. a) silk fibre, b) silk fibroin, c) fibrin, d) bovine serum albumin, e) human serum albumin, (10^5r) and e') human serum albumin (10^4r).

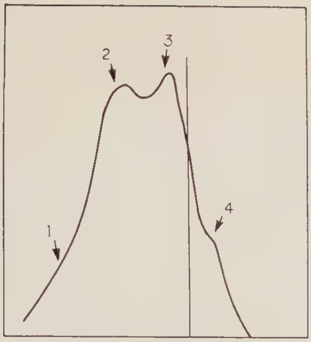

Fig. 3-B. Integrated pattern of ESR signal (sample: reduced glutathione).

The numbered arrows pointing downward show g=2.04 (1), 2.027 (2), 2.017 (3), and 2.003 (4) respectively.

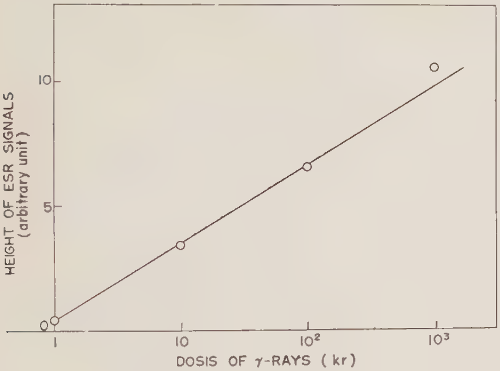

Fig. 4. Relationship between the radiation dose of γ-rays and height of the ESR absorption spectra of irradiated bovine albumin.

Abscissae: Dosis of γ-rays.

Ordinate: Height of ESR signals. (arbitrary unit)

is transferred to the S–S or –SH bond through a certain mechanism, the ESR spectra of cystine type being obstained.

When the magnetic field was applied perpendicular to the fibre axis, the spectra of silk fibre was a symmetrical doublet with a splitting of about 10 gauss, while fibroin powder provided a singlet with g-value near 2.003. Such a difference would be an orientation-dependent effect, since the former has an elongated polypeptide chain structure with the adjacent chains linked by hydrogen bonds between the C=O and NH groups, but random orientation of such a structure occurs in the latter.

Effect of Oxygen—As reported in the preliminary reports (*5*), the ESR signal of albumin irradiated *in vacuo* decreased its height and disappeared when exposed to air, while that irradiated in the air could not be detected a week or more after irradiation. Hence irradiation of albumin was made in the presence of O_2 and N_2. As shown in Fig. 5b, the pattern quite different from that irradiated *in vacuo* (Fig. 5a) was obtained when irradiated in O_2, while the presence of N_2 causes hardly any alteration of ESR signal. Since the sensitivity of spectrometer had to be increased to detect the distinct signal for albumin irradiated in O_2 (4.5 times), augmented zero line drift and background noise were inevitable as seen in the illustrated record and the absorption curve obtained by integrating the original records is not to be very accurate. It is very likely from Fig. 5b, however, that the absorption spectra in O_2 is a singlet, its peak giving g-value of about 2.003, while that *in vacuo* is asymmetrical one, quite similar to that of cysteine or cystine, and composed of some components whose g-values are differnt from 2.003 (see above).

Similar observation were made on fibrin, its ESR signal being somewhat similar to that of human serum albumin when irradiated *in vacuo*. When irradiated in O_2, its signal also could be detected only by raising the sensitivity of apparatus. Though its record is not so satisfactory one because of augmented zero line shift and background noises, it seems to be a slightly asymmetrical singlet with g of about 2.003, which resembles that of bovine albumin.

From these results, therefore, the ESR signal obtained on the protein irradiated in the air should be attributable to the oxide radicals formation as pointed out by Gordy and his co-workers (*10*) or known on the irradiated synthetic high polymers (*1*).

Effect of Water—Effect of the presense of water was observed on the paste-like state of bovine serum albumin prepared by introducing vapour-saturated air into the sample

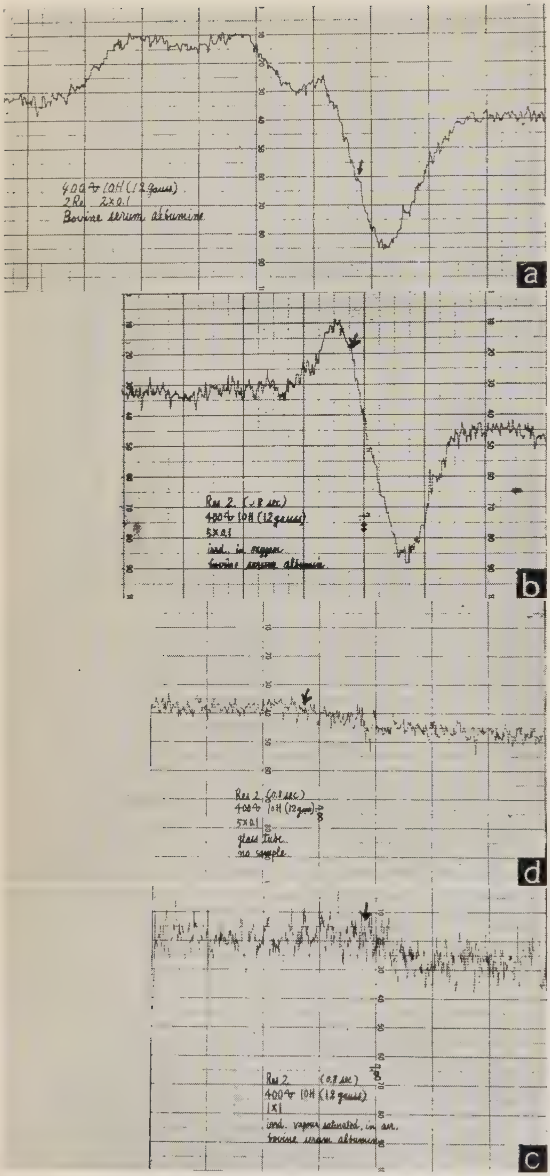

FIG. 5. ESR absorption spectra of γ-irradiated protein in the presence or absence of air and water.

a: Bovine serum albumin (in the absence of air).
b: Bovine serum albumin (in the presence of air).
c: Bovine serum albumin (in the presence of water).
d: Glass background signal.

tube or by adding the small quantity of water at reduced pressure. In these cases, observations were made on the tube thinner than the usual one (about 1/2 in its diameter). When irradiated by 10^7 r γ-rays in such a state, samples became gel-like and their signal obserbed was very small, even when the sensitivity of spectrometer was elevated (about 5 times), accurate tracing of absorption spectra being difficult because of their smallness as well as the presence of increased noise. Comparing with the control background signals of the sample tube alone (Fig. 5d), ESR signal may exist in the record of Fig. 5c, even only in the slightest extent. Such a great reduction or disappearance of ESR absorption due to the presence of water is not attributable to increased dielectric loss in the cavity, since the power received by the crystal detector was kept at nearly the same level as in the observation in the absence of water by adjusting its coupling hole. Moreover, the paste prepared immediately before the recording by adding water to the bovine albumin irradiated *in vacuo* provided a distinct signal similar to that in the dry state, even though its height decreased. Since the observation on the paste were usually made 24 hours or more after irradiation, however, it is imppossible to say conclusively about the nature of free radicals formed immediately after irradiation in the presence of water. Free radicals from irradiated water molecules would probably play an important role as sugessted by the previous experiments on titrable SH group of irradiated serum albumin (*5*).

Titration of SH Group of Iradiated Albumin—Using argentinometric titration, amount of SH group in the albumin solution after adding irradiated albumin or amino acids was measured, its procedure of titration being quite similar to that in the previous report (*5*) except that an automatic potential recorder was used instead of a galvanometer (Tris buffer of pH 7.8 with vibrating platinum electrode). When the titrable sulphhydryl group of irradiated bovine serum albumin was measured immediately after dissolution in the buffer of its irradiated solid sample (10^7r), the values obtained was slightly higher than that of non irradiated one. Such an increase can not be attributed to a reaction of Ag^+ ion with free radicals formed in the residues other than cysteine or cystine, because irradiated amino acids or peptides free from —SH or -S-S- group never consumed a significant amount of Ag^+ ion, while the irradiated cysteine gave results similar to that of irradiated albumin. The titrable —SH group of irradiated albumin as well as cysteine diminished slowly and it is probably due to interactions between free radicals formed on SH.

Immediately after dissolution of the irradiated albumin (or amino acids), solution of native albumin of equivalent concentration was added in various propotions and then the mixture was titrated promptly. An example of results obtained in such experiments was illustrated in Fig. 6, in which SH group content of native or irradiated protein (or amino acids) alone was represented by the points N and I respecttively.

If interactions between both proteins never affects SH group, the observed points would fall near the straight line NI. But it is not the case, amount of titrable SH group of the mixture being evidently lower than their expected value (Fig. 6B). When the solution of irradiated albumin was replaced with that of irradiated cysteine (10^6r), the result was quite similar to the above stated one, while SH group of native albumin appeared almost unaffected when the solution of irradiated aliphatic amino acids such as glicyne and leucine, or of phenylalanine, histidine, proline, tryptophan and peptides such as glycylleucine and glycyl-glycyl-glycine was used instead of irradiated cysteine solution (Fig. 6B). As shown in Fig. 6A, circumstance were quite similar, when heavily irradiated cysteine (10^6r) was added to the native cysteine solution. In contrast to this, weakely irradiated cysteine (10^4r) gave a low titrable SH content and it produced a linear reduction of SH content indicating only a dilution effects of mixing, when added to the native cysteine solution. On the other hand, addition of irradiated (10^6r) cystine to the native cysteine also produced a reduction of SH content in a linear fashion, a fact which suggests that -S-S- free radical is not involved in such a

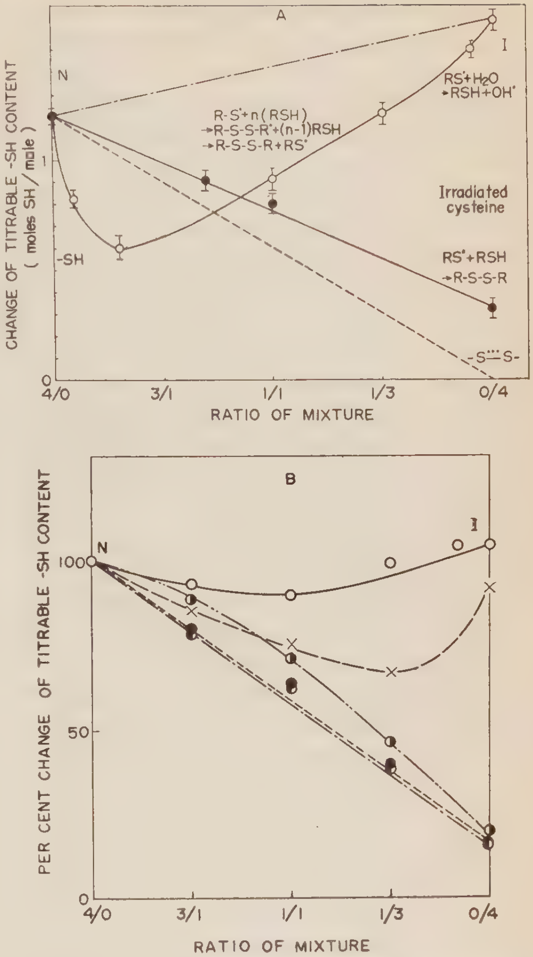

Fig. 6. Changes in titrable -SH content in the mixture of the native substances (N) with irradiated ones (I).

A. Abscissae: ratio of the mixture (N and I).
N: Native cysteine
I: irradiated cysteine (○; 10^6, ●; 10^4) and cystine (....) 10^6r.
Ordinate: change of titrable -SH content.

B. Abscissae: ratio of the mixture (N and I).
N · native bovine serum albumin.
I: 10^6r irradiated protein (○), glutamic acid (●), prolyne (◐), cysteine (×) and tyrosin (◐).
Ordinate: % change in titrable -SH content. (Each experiment was performed immediately after dissolution.)

reduction in titrable SH group. In weak irradiation, amount of free radical produced (RS·) would be only small and titrable SH group would be probably disappeared by such a reaction as (1) $RS\cdot + RSH \longrightarrow R{-}S{-}S{-}R$, while the reaction (2) $RS\cdot + H_2O \longrightarrow RSH + OH\cdot$ would provide titrable SH group when heavily irradiated. The gradual reduction of titrable SH group of heavily irradiated cysteine and albumin would be explained by combined effect of reaction (1) and (2). When native cysteine or albumin is present, a reaction such as (3) $RS\cdot + n(RSH) \longrightarrow R{-}S{-}S{-}R\cdot + (n-1)RSH$ occurs, from which decrease in titrable SH group illustrated in Fig. 6 would result.

The mechanism of such a reduction in titrable SH group would be not so simple, and above-mentioned mechanism is only a speculative one but it seems obvious that a free radical formed in –SH group by ionizing radiations plays an important role in this reaction, amino acids other than cysteine being not involved. The above-mentioned effect of irradiated albumin on SH group content should be considered, therefore, to provide an indirect support to a view, as the results of ESR absorption studies, that free radical formation in the albumin molecules occurs chiefly, though not entirely, on their cysteine or cystine residues. The systematic observation on such an effect of irradiated SH group is now going on in our laboratory.

DISCUSSION

Since 1956, Gordy and his associates have studied on the ESR spectra observed from various X-irradiated proteins and tried to compare them with those obtained on the simpler peptides and amino acids. As stated by Ingram (*1*), such a method of analysis by "correlation of similar spectra" is most feasible and most appropriate one at the present stage of biological ESR studies, even though the conclusion deduced in this way would be considererd as only tentative. The results obtained from the proteins, amino acids and peptides irradiated by γ-rays are, as shown above, nearly identical with those of Gordy and his coworkers.

The ESR patterns of γ-ray irradiated amino acids can be classified into three categories accordings to their g-value, splitting and symmetricity (*6*):

1) Aliphatic amino acids: the pattern is usually composed of nearly symmetrical 3, 5 or more lines according to the shape and length of carbon chain, its g-value being nearly 2.00 and its over-all splitting is more than 90 gauss except glycine (about 80 gauss).

2) Amino acids containing aromatic ring: their g-value is also near 2.00, but their over-all splitting is narrower (less than 80 gauss) than the former and the line width is usually narrow. Such a narrower spacing might be originated from the motional narrowing due to the ring π-electron systems. Tryptophan also gives a similar pattern, but the pattern of proline and hydroxyproline is different, for they have no conjugated double bonds and so no mobile electrons.

3) Cysteine and cystine: their pattern is markedly asymmetrical and appears to be composed of some components whose g-values are considerably different from 2.00.

As stated in the previous section, proteins and peptides containg sulphhydryl groups provide a ESR pattern quite similar to that of the category 3 in the above classification, while fibroin and peptides composed of aliphatic amino acids show the pattern similar to the category 1.

The triplet obtained on the irradiated glycine is quite similar to that reported by Gordy *et al.* These investigators attributed it to a $C{\cdot}H_2$ radical formed, which is then stabilized by attaching to an adjacent RO^- ion. Similarly, the quintet from the irradiated alanine was postulated to be originated from $(CH_2 \dot{-} CH_2)^+$ group (*2*).

Recent observation on the single crystal of glycine (*11*) or of alanine (*12*) revealed, however, that the hyperfine structure of these compounds was not always so simple as postulated from the previous results obtained from the powdered samples. So the above reasoning are only tentative, more detailed and accurate analysis being required.

The doublet of acetyl-glycine would be assumed tentatively to arise from ($CH_3CO \cdot CH^{\cdot}$) and that of glycyl-leucine probably from C_4H_9-($NH_2 \cdot CH_2 \cdot CO \cdot NH$)$\cdot CH^{\cdot}$. It seems worthy to note here that the pattern of glycyl-glycyl-glycine rather resembles a singlet of indented peak. This would be partly explained by the presence of small amount of radical species such as glycyl-glycyl-$NH \cdot CH_2 \cdot COO^{\cdot}$ or $-C=O^{\cdot}$.......H–N–, in which an unpaired electron mainly localized on an O-atom. Similarity of the pattern of silk fibre to that of this tripeptide suggests that the orientation-dependent doublet of the former is ascribed to an odd electron localized on an O-atom experiencing direct dipole interaction with the hydrogen-bonding proton as postulated by Gordy *et al.* (*2*). On the other hand, fibroin powder gives only an asymmetric singlet because of smearing out of such an effect by random orientation of polypeptide chain structure.

Concerning the patterns of serum albumin and of polypeptides containing sulphhydyl group, their resemblance to the characteristic asymmetrical spectra of cysteine or cystine is quite remarkable, a fact which strongly suggests that such spectra could be explained as due to an unpaired electron localized on the –S–S– bond or –S radical in the molecule. Their close resemblance to the spectra of polymeric sulphur in oleum as well as the observations on the changes in titrable –SH group of native albumin and some amino acids also afford indirect support to this view.

Gordy *et al.* supposed that the two sulphur atoms of the cystine molecule acts as an electron reservoir, supplying electrons to fill vacant holes at other positions in the molecule produced by irradiation and thus an additional low-energy three electron bonding is formed. Thence the loss of an electron from the sulphur lone pair orbitals occurs, which is assumed to strengthen rather than weaken the bond. But any character of semiconductor has been never found on the serum albumin, nor proof on the low-energy of three electron bonding has been afforded hitherto. In one preminary experiments on the single crystal of irradiated cysteine and thioctic acid, an evidence on anisotropy of their g-values was obtained (to be published), which seems difficult to be interpreted even from such a view-point. To explain the peculiar pattern of irradiated cysteine or cystine thoroughly, therefore, further studies, experimental and theoretical, should be required.

Putting aside validity of their interpretation, however, the radiation-induced free radicals in the proteins seem to be associated with their cysteine and cystine content. In the pattern of human serum albumin, the component of $g=2.003$ is more pronounced than in bovine one and it is overwhelmingly predominant in the fibrin. As reported previously, the irradiated alanyl-phenylalanine provided ESR spectra, in which both patterns of aromatic and aliphatic amino acids (category 1 and 2) were mixed (*6*). And so the above result would be also explained as a similar mixing of the pattern of aromatic ring to that of cystine type, for the line width of this component seems relatively narrower.

The oxygen effect was noticed already in the initial ESR studies on polymers and also found on irradiated protein (*5*). The adsorption of molecular oxygen was considered to form a transient R–O–O$^{\cdot}$ radical with the initial free radical group. This peroxide radical breaks up into $HO_2^{\cdot}$ or similar radicals which react to give non-radical product and so a gradual disappearance of initial signal. Otherwise, this peroxide radical remains stabilized and trapped. The results observed on the serum albmin and fibrin indicate that the oxygen effect on these proteins is the former. Usually the unpaired electron is strongly localized on the O-atoms and so its spectra would be very anisotropic, for the resultant complex can be regarded as having a three electron bond between the two oxygen atom just as in the above-stated S-S bond. In the amorphous sample, however, such an anisotropy is smeared out, the relatively wide singlet being obtained. This is just the case for albumin and fibrin. It should be noted, however, that the three electron bond of S–S radical and peroxide radical is quite similar in their nature, but the former gives a peculiar asymmetric pattern even in the amorphous powder and the latter not. (This might be also considered as a fact unfavourable to the three electron bond concept.)

Radicals formed by radiation in the presence of water could not, as stated above, be examined. As reported previously (*5*), the titrable SH group of albumin decreased promptly after irradiation under the dissolved state even in the absence of O_2, while that in the solid state remained unaltered for 10^4r of γ-rays. Moreover, irradiated albumin interacts with the SH group of native protein or cysteine immediatery after its dissolution, and produces remarkable reduction of titrable SH content of the latter two. Henceforth, it seems highly probable that the effect of radiation on the protein solution is chiefly, if not entirery, attributable to irradiated water, and the decrease in the SH group of irradiated protein solution should be partly ascribed to such a free radical reaction. A fact that any ESR signal could be hardly obtained in the presence of water would be also explained from such a point of view, but prompt disappearance of –S–S– or –S radical taking place in water medium might be partly responsible for it.

SUMMARY

1. Investigations on the ESR absorption spectra of γ-ray irradiated bovine and human serum albumin, fibrin and silk fibroin were made.

2. The free radicals in the irradiated proteins are associated with their cystine or cysteine content, the higher their content of these sulphur-containing amino acids, the more resemble their ESR patterns that of cystine or cysteine.

3. Silk fibre provided a doublet quite similar to that of glycyl-glycyl-glycine. Such an orientation-dependent pattern would be explained as an unpaired electron localized on the oxygen atom hydrogen-bonded to NH group of the adjacent polypeptide chain.

4. When irradiation was made in the presence of oxygen, the ESR signals of proteins are remarkably different from those irradiated *in vacuo*, the former being attributable to peroxide radicals.

5. The proteins irradiated in the presence of water could barely provide a noticeable ESR signal.

6. When irradiated albumin, or cysteine were dissolved into the solution of native albumin, the titrable –SH group decreased, probably due to reaction of sulphhydryl radicals with the –SH group.

From these results some considerations were made on the free radical formations in the irradiated protein.

The author wishes to thank to Prof. A. Inouye for his valuable discussion, and Dr. H. Honda. for his kind permission to use the ESR spectrometer (V-4500) and Mr. S. Toyoda and Dr. A. Hirai for their valuable assistance to perform this experiment.

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The Spectrophotometric Determination as Trinitrophenyl Derivatives of Amino Acids and Peptides Resolved on Paper by Electrophoresis and Chromatography[*]

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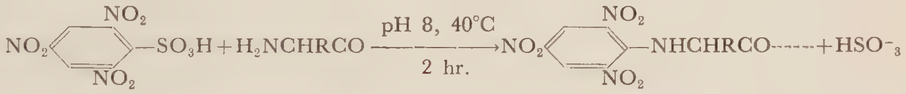
The amino acid composition of peptide- or protein-hydrolysate has been determined within an error of 3 per cent by ninhydrin colorimetry of chromatographic effluent from a column of Amberlite IR 120, according to the method developed by Moore, Stein and Spackman (*1*). More conveniently but with less accuracy, it has been also analyzed by paper chromatography and the ninhydrin colorimetry of each spot (*2*), or by quantitative dinitrophenylation, paper chromatography of the resulting dinitrophenyl amino acids, and spectrophotometry of the extract of each yellow spot (*3*). In the latter case, however, minute resolution of a complicated mixture of dinitrophenyl amino acids has been achieved only with difficulty, as compared with that of free amino acids; while the ninhydrin colorimetry of free amino acids on paper, in the former case, with much less accuracy than the direct spectrophotometry of dinitrophenyl amino acids.

As already reported by the present author (*4*), amino acids and peptides content in solution (0.01— to 1 μmole) is able to be determined spectrophotometrically with an error of 2 per cent, after the quantitative TNP-ation with TNBS (*5*). Such a TNP-ation of amino acid or peptide with TNBS also occurs on filter paper, and a spot of amino acid or peptide produces an intense yellow color by spraying slightly alkaline TNBS solution, although the TNP-ation is far from quantitative.

Recent studies in this laboratory indicated that a spot of amino acid or peptide so colored with TNBS, could be eluted and further TNP-ated quantitatively, merely by dipping each cut spot into TNBS solution at pH 8 and at 40°C (*4, 5*). In this paper will be described an application of this TNBS technique to the quantitative determination of minute amount of amino acid and peptide resolved on paper by electrophoresis and chromatography.



EXPERIMENTALS

Materials—TNBS, beef insulin (24 unit per mg.), authentic TNP-amino acids and standard amino acids used in this study, were the same ones as used in the previous papers (*4, 5*), respectively. Bovine globin α and β were obtained from the whole globin by fractional precipitation of the β-chain with N trichloroacetic acid in the presence of 8 M urea, according to the method of Hayashi (*6*).

Preparation of Sample to Be Analyzed—Standard solution of known amino acid mixture was obtained by mixing a definite volume of amino acid solution of a known concentration (10 to 100 μmole per ml.). As the solvent, $N/100$ hydrochloric acid was used, except in the cases of cystine and tyrosine which were dissolved into $N/10$ hydrochloric acid, respectively.

Acid hydrolysate of protein was prepared by heating 5 to 10 mg. portion of protein with twice redistilled 5.7 N hydrochloric acid (5 ml.) in a sealed tube at 110°C for 24 hours, by repeated evaporation of the resulting hydrolysate over sodium hydroxide in an evacuated vessel, and dissolving the residue into a definite volume of distilled water (0.1 ml.; the final pH being about 2).

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[*] Following abbreviations were used: TNBS for 2,4,6-trinitrobenzene-1-sulfonic acid, and TNP for 2,4,6-trinitrophenyl.

An equimolecular mixture of oxidized insulin A and B was obtained by performic acid-oxidation of beef insulin, according to the method of Sanger (*7*). The resulting solution was directly resolved by paper electrophoresis (*8*).

Resolution of Amino Acids and Peptides—A mixture of peptides (oxidized insulin A and B) was separated one-dimensionally on a paper stript of 5×40 cm. by electrophoresis, while a mixture of amino acids (synthetic mixture and acid hydrolysate of protein) was resolved two-dimensionally on a paper sheet of 40×40 cm., by electrophoresis then by chromatography (see Fig. 1).

Otherwise mentioned, paper electrophoresis was performed at a potential gradient of 30 volt. per cm., using a mixture of pyridine (60 ml.), acetic acid (1.8 ml.) and water (940 ml.), pH 6.65 as the medium. A mixture of 1-butanol, acetic acid and water (freshly prepared, 25:6:25, v/v) was the solvent system of chromatography. The resolution of leucine and isoleucine was achieved by multiple development with this solvent (*2*).

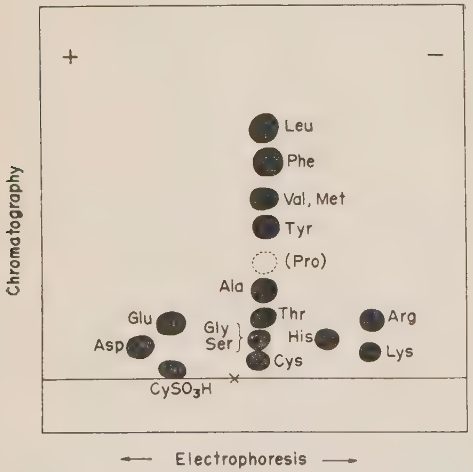

FIG. 1. Two-dimensional resolution of amino acids.

Electrophoresis: Pyridine-acetic acid-water, pH 6.65, 900 volt., 60 minutes.

Chromatography: 1-Butanol-acetic acid-water (25:6:25, v/v).

Valine/methionine and glycine/serine were separated only partially by this method.

Proline did not colored with TNBS.

Detection of Spot Resolved on Paper—After the separation of amino acids and peptides, the paper was dried as soon as possible under an air current at 45° to 50°C, to avoid their diffusion on a wet paper. The paper developed with acetic acid-containing solvent, was further dried in an oven at 60°C, to remove a trace of the acid. Thereafter, the paper was sprayed only slightly with 0.1% TNBS in 80% methanol, then with methanolic solution of borate-phosphate buffer ($M/5$ $Na_2B_4O_7$-$M/2.5$ KH_2PO_4 in 70% methanol, pH 8.5) to make the paper to be slightly alkaline. Almost immediately spots of basic amino acids produced intense yellow color due to the TNP-ation. On standing the paper in a dark place (as TNP-derivatives being photosensitive (*4*, *5*)) at room temperature, neutral and acidic amino acids also colored within 5 to 20 minutes, respectively.

Quantitative Determination of Each Colored Spot—Each yellow spot so colored, was cut off from the paper, including the surrounding area with a width of 3 mm. Each spot was further cut into smaller pieces, was placed into a test tube, and was added with one ml. of 0.1% TNBS in water and with 2 ml. of $M/10$ borate buffer, pH 8.0.

The spot so dipped completely into the bufferized TNBS solution, was kept for 2 hours in a dark place at 40°C, for the additional TNP-ation and extraction from paper. Thereafter, a definite amount of $2N$ hydrochloric acid (usually 1 ml.) was added and the optical density of the solution was determined at 340 mμ (*4*, *5*) with the Shimazu Spectrophotometer Model QR-50. In the cases of lysine, cystine and higher peptides, whose TNP-derivatives were only slightly soluble in aqueous hydrochloric acid, was used methanolic hydrochloric acid or glacial acetic acid, instead of aqueous hydrochloric acid. As a control, use was made of a piece of blank paper (5 cm^2.) which had been sprayed with the both solutions, and treated just the same as mentioned above. The optical density of blank, however, was usually as low as 0.03 to 0.05 at 340 mμ.

RESULTS AND DISCUSSION

Detection of Spots of Amino Acid and Peptides—By spraying slightly alkaline TNBS solution, spots of amino acid and peptide on paper produced an intense yellow color, except proline and prolyl peptide which had been elucidated not to react with TNBS (*4*). As already reported by the present authors (*4*) the higher the pH, the greater the velocity of coloration, but there produced an intense coloration of background, which caused troubles in the spectrophotometry of each spot.

Under the conditions described in experimental part, the limit of detection was approximating, to 0.01 μmole for basic and neutral amino acids, and 0.02 μmoles for acidic ones. These values were rather similar to those obtained by coloration with ninhydrin. In the case of higher peptide, however, this TNBS method was much more sensitive than the ninhydrin method.* The result suggested that TNBS could be used for the coloration of peptides on finger print map, instead of less sensitive ninhydrin (*9*, *10*).

Spectrophotometric Determination of Spots—The coloration of amino acid- and peptide-spots with TNBS seemed to be due to their TNP-ation, as the extract of each yellow spot had an ultraviolet absorption spectrum (280 to 430 mμ.) essentially the same as that of the corresponding TNP-derivatives (*5*). The magnitude of optical density, however, indicated that the reaction on paper is neither quantitative nor reproducible.

On further treatment of each cut spot with bufferized TNBS solution (1.0 ml. of 0.1% TNBS and 2.0 ml. of *M*/10 borate buffer pH 8.0). Under the standard conditions (2 hours in a dark place at 40°C) (*4*), the reading of optical density increased up to 97 to 102 per cent of the theoretical value. Fig. 2 illustrates the optical densities so obtained and the amount of amino acid applied on paper. The result indicated that the readings were accurately propotional to the amounts, and that the leucine equivalent of a given amino acid was just the same as the ratio of molar extinction coefficient of the TNP-ated sample against that of TNP-leucine. Table I summarizes the leucine equivalents of vorious amino acids assayed by this TNBS technique, after their two-dimensional resolution by electrophoresis and chromatography (see Fig. 1), together with their theoretical ones calculated from the molar extinction coefficient of authentic TNP-amino acid (*4*, *5*). The both values were very close in all amino acids, except aspartic and glutamic acids. Their lower leucine equivalents seemed to be due to the use of acetic acid as the solvent for chromatographic resolution.**

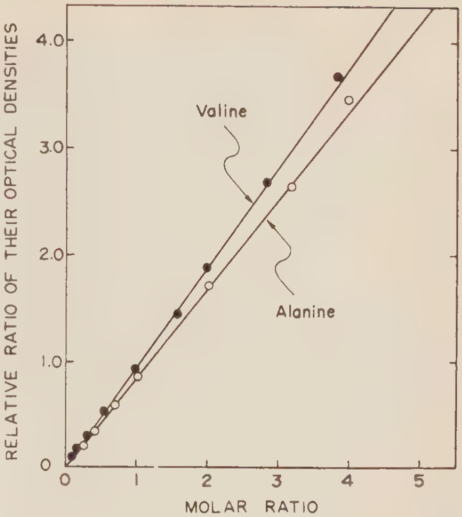

Fig. 2. Relation between the optical density and amino acid content.

Mixtures of leucine and valine or alanine whose content ratios were shown on the abscissa, were separated by paper chromatography, and the relative ratios of their optical densities shown on the ordinate, were measured.*

The leucine equivalents of valine and alanine were 0.96 and 0.83, while their theoretical ones were 0.94 and 0.86, respectively (*4*).

Amino Acid Composition of Protein—Table II and III summarize the amino acid composition of insulin and of the fractionated globins assayed by this TNBS method. In these experiments, acid hydrolysate of protein was resolved two-dimensionally by electrophoresis and chromatography as described in experimental part (see Fig. 1), and leucine equiva-

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* Molar extinction coefficient of TNP-peptide was in a range of 1.0–1.1×10⁴ (*4*, *5*). This value was similar to those of TNP-amino acids.

* The optical densities were proportional to the actual amount of sample applied on paper. However, the use of internal marker method (leucine being the marker in this case) gave more accurate results.

** It was because that their values, if the previous resolution was achieved with the use of other solvent systems (2-butanol: water=4:1, v/v; pyridine: water=4:1, v/v), were very close to the theoretical ones, respectively. The reason has not yet been elucidated.

TABLE I

Leucine Equivalents of Various Amino Acid in TNBS-Method.

Amino acid	A	Max. dev.	B	A~B%
Glycine	0.85	0.02	0.87	2
Alanine	0.84	0.02	0.86	2
Valine	0.96	0.01	0.94	2
Leucine	1.00	0.00	1.00	0
Isoleucine	1.02	0.01	1.03	1
Serine	0.93	0.01	0.95	2
Threonine	0.96	0.02	0.95	1
Aspartic acid	0.70	0.01	0.80	10[1]
Glutamic acid	0.87	0.02	0.94	6[1]
Cystine	0.83	0.04	0.84	2
Cysteic acid	0.91	0.03	0.92	2
Methionine	0.96	0.01	0.94	2
Methionine sulfone	0.94	0.01	0.92	2
Tyrosine	0.99	0.03	0.96	4
Tryptophan	0.80	0.03	0.83	3
Phenylalanine	0.97	0.02	0.95	2
Histidine	0.96	0.02	0.94	2
Arginine	0.98	0.02	1.01	3
Lysine	0.85	0.02	0.88	3

A: Observed values by the present method.

B: Theoretical values calculated from molar extinction coefficients of TNP-amino acids (*4*).

1) If the resolution was achieved with neutral or basic solvent, their values were very close to the theoretical ones, respectively.

TABLE II

Amino Acid Composition of Insulin

(Expressed as the numbers of residues per mole)

Amino Acid	Found		Analyzer	Theor.
	a	b		
Lysine	1.03	1.00	1.00	1
Histidine	1.86	2.05	1.89	2
Arginine	0.96	1.01	0.98	1
Aspartic acid	2.87	3.01	3.02	3
Glutamic acid	6.83	6.70		7
Cystine/2	4.88	5.75	5.27	6
Glycine } Serine	6.71	6.70	3.91	4
			2.63	2.7[1]
Threonine	0.98	0.97	0.96	0.95[1]
Alanine	3.08	2.89	2.44	3
Tyrosine	3.35	3.45	3.75	4
Valine	5.14	4.82	4.38	5
Isoleucine	1.01	0.83	0.58	1
Leucine	6.30	6.21		6
Proline	[2]	[2]	0.87	1
Phenylalanine	2.93	3.07	2.88	3

a, b) Two independent experiments.

1) No correction on the destruction of serine (10%) and threonine (5%).

2) Proline did not colored with TNBS.

lents shown in the column A of Table I, were used for the calculation of amino acid contents from their optical densities. Two pairs of amino acids, valine and methionine, and glycine and serine, were resolved from each other only partially in this cases, and proline did not react with TNBS (*4*, *5*). As far as the other amino acid compositions were concerned, however, the result on insulin was in good agreement with the theoretical one, and the accuracy seemed to be as high as ±4 per cent.

Relatively low recoveries of isoleucine and tyrosine from insulin will be due to the difficulty in hydrolysis of isoleucine- and tyrosine- containing linkages. It was because that some amounts of peptides* which had been scarcely detected with ninhydrin, were observed by coloration with TNBS. According to Kotaki**, this method was effective for the studies on the genetically determined differences among the amino acid compositions of various insulins.

The amino acid compositions of bovine globins α and β were fairly similar to those of human globins α and β, which had been analysed by Craig (*11*) with the method of Moore, Stein and Spackman (*1*), respectively. Each sum of the numbers of a given amino acid residue in bovine globins α and β, was also not inconsistent with those of the unfractionated bovine globin reported by Huisman (*12*).

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* Two unidentified spots were observed near the positions of phenylanine and leucine.

** Unpublished (Kotaki, A.)

TABLE III

Amino Acid Composition of Fractionated Bovine Globins

(Each amino acid content was expressed as the numbers of residue per mole)

Amino acid	α Chain		β Chain	
	Bovine	Human (*11*)	Bovine	Human (*11*)
Glutamic acid	6.9	5	9.9	10
Aspartic acid	11.8	11	15.7	13
Lysine	10.1	10	11.1	10
Histidine	7.1	10	7.9	10
Arginine	3.0	3	4.0	4
Cysteine	0.8	1	1.3	2
Glycine	19.3[1]	6	18.3[2]	6
Serine		9		5
Threonine	7.3	8	7.8	7
Alanine	18.1	19	19.2	14
Tyrosine	1.7	3	1.9	3
Valine	11.6	12	15.6	18
Methionine	1	3	2	1
Phenylalanine	7.1	7	7.9	8
Tryptophan	1.0[3]	1	1.0[3]	3
Isoleucine	0.0	0.0	0.0	0.0
Leucine	16.1	16	18.8	19
Proline	4.5[4]	7	6.6[4]	7
Total	127.4	132	150.8	139

1) According to Levy's method (*15*), 9.0 for glycine and 10.3 for serine.

2) According to Levy's method (*15*), 11.2 for glycine and 7.1 for serine.

3) Determined by the direct spectrophotometry on intact protein.

4) Determined with Levy's method.

Spectrophotometric Determination of Peptides—As already reported by Satake (*4*, *5*) molar extinction coefficients of TNP-peptides were similar to each other in their magnitudes (1.05×10^4) and in their wave length at the maximum absorption (340 mμ in an acidic medium), from simple dipeptide up to higher one such as oxidized insulin. These facts seemed to indicate that this TNBS method could be also used for the assay of peptides resolved on paper (*9*, *10*), if the TNP-ation and the extraction from paper were quantitative. Fig. 3 shows the paper electrophoretic pattern of an equimolecular mixture of oxidized insulin A and B. The ratio of the readings on the both components was 1 : 1.57 by the ninhydrin densitometry and was 1 : 1.92 by this TNBS method. The latter value was very too close to the ratio of amino contents of the both components (1.0 : 2.0) and to the ratio of molar extinction coefficients of the both TNP-derivatives (1.03 : 2.01 = 1 : 1.94).

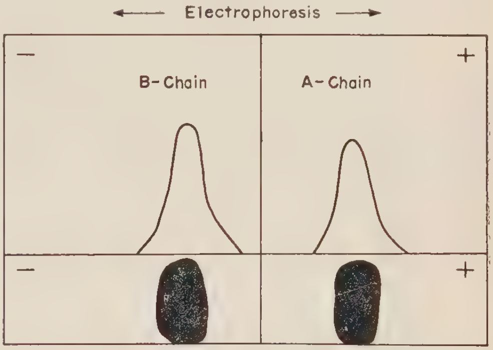

FIG. 3. Paper electrophoretic patterns of oxidized beef insulin.

Directly densitometried after staining with ninhydrin (*13*).

In connection of these peptide analysis with TNBS, it should be emphasized that each TNP-ated peptide, after the spectrophotometry, might be used for the purpose of several structural analyses, such as N-terminal and non N-terminal amino acid analyses, or might be regenerated to the original peptide merely by treatment with ammonia at room temperature, without acompanying any splitting of their peptide bounds (*14*).

$$\text{TNP-NHCHRCO-NHCHR'CO}\cdots \xrightarrow[100^{\circ}\text{C},\ 10\ \text{min.}]{15\,N\ \text{NH}_4\text{OH}} \text{Picramide}+\text{H}_2\text{NCHRCO-NHCHR'CO}\cdots$$

Such procedures have been used with successes by the present authors on the structural analyses of peptides derived from the fractionated bovine globins with proteinases. These results will be soon published elsewhere.

SUMMARY

1. Amino acid and peptide resolved on paper by electrophoresis and chromatography (0.01 to 0.03 μmoles per cm^2.) could be detected as yellow spots by TNP-ation with TNBS.

2. Each spot so colored with TNBS (0.01 to 0.5 μmoles) could be determined within an error of ±3 per cent by dipping the cut spot into TNBS solution bufferized at pH 8 to complete the TNP-ation, then acidification, and spectrophotometry of the resulting supernatant.

3. Using this TNBS technique, amino acid compositions of bovine insulin, globins α and β were determined.

4. This method could also be used with success for the quantitative determination of peptides, as molar extinction coefficients of their TNP-derivatives were of similar magnitude (1.05×10^4).

The authors are indebted to Miss M. Yoshida for her technical assistance.

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Biochemical Studies on Sulfate-Reducing Bacteria

X. Adenosine-5′-Phosphosulfate Reductase

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Sulfate reduction is one of the most important pathways of metabolism not only as an assimilation process of inorganic sulfur in higher plants and many kinds of microorganisms but also as an energy-yielding reaction in sulfate-reducing bacteria. However, the enzymic mechanism of this process has not been confirmed until recently because of the failure in preparing cell-free systems. Wilson and Bandurski (*1*) and Hilz and Kittler (*2*) observed the sulfate reduction in yeast extracts in the presence of adenosine triphosphate and suggested activation of sulfate leading to the formation of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) which was in turn reduced to sulfite. It was found by the present authors that sulfate was reduced stoichiometrically to sulfide in extracts of a sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, in the presence of ATP (*3*), and participation of adenosine-5′-phosphosulfate (APS) as an intermediate was stated from the facts that APS was produced from ATP and sulfate in the extracts and that APS synthesized chemically was reduced to sulfide and adenylate (*4*). The following reactions were proposed as the pathway of sulfate reduction in *D. desulfuricans*.

$SO_4^{--}+ATP=APS+\text{pyrophosphate}$
$APS+H_2=AMP+SO^{--}_3$
$SO^{--}_3+3H_2=S^{--}+3H_2O$

Peck has also presented the same scheme from the identification of APS and observation on the formation of pyrophosphate and sulfite from ATP and sulfate in extracts of *D. desulfuricans* (*5*).

In this communication studies on partial purification and properties of APS reductase are reported. Preliminary communications have already appeared (*6*, *7*).

MATERIALS AND METHODS

Preparation of Crude Extracts of Sulfate-Reducing Bacteria—The strain used and conditions of culture were the same as described in a previous paper (*8*). Cells were disintegrated by sonic oscillation (10 KC). The sonicate was centrifuged at 18,000×*g* for 15 minutes. The supernatant solution was used as the crude enzyme preparation in the following experiments.

Preparation of Hydrogenase—The precipitate (18,000 ×*g*, 15 minutes) from the sonicate was washed twice with 0.17% sodium chloride solution, suspended in 0.05 *M* phosphate buffer, pH 7, and employed as the hydrogenase preparation (9). This preparation had high hydrogenase activity and was free from APS reductase.

Materials—AMP and ATP were the products of Sigma Company. Cytidylate, uridylate and guanylate were gifts of the Takeda Pharmaceutical Co. S^{35}-sulfite was prepared by Dr. H. Shimojima.

Preparation of Nucleoside Phosphosulfates—APS was prepared according to the method of Baddiley *et al.* (*10*). Synthesis of guanosine phosphosulfate (GPS), uridine phosphosulfate (UPS) and cytidine phosphosulfate (GPS) were carried out respectively from GMP, UMP and GMP by the similar method. These preparations gave similar spectra to those of the corresponding mononucleotides. Sulfate contents were determined by the benzidine method (*11*) after 7 minutes heating at 100°C in *N* HCl. Molar contents of sulfur were proved to be equal to that of bases determined spectrometrically. By paper electrophoresis in ammonium acetate buffer, pH 5.5, each nucleotide was found to migrate as a single spot at a faster rate than the corresponding mononucleotide.

Assay of Reductase Activity—Reduction of nucleoside phosphosulfate, thiosulfate, and sulfite was carried out in Warburg manometers in an atmosphere of hydrogen. The system consisted of a substrate, the enzyme preparation, a sufficient amount of hydrogenase and methylviologen as an intermediary electron carrier. Reaction mixtures contained 50 μmoles of phosphate buffer, pH 7.0, 1 μmole of methylviologen, enzyme solution, hydrogenase (0.7 mg.) and 1 μmole of APS or 5 μmoles of thiosulfate or 5 μmoles of sulfite in a total volume of 1.6 ml. Center wells contained 0.2 ml. of $2N$ KOH. Gas phase was hydrogen and temperature was 30°C. Under these conditions, the velocity of hydrogen uptake was approximately proportional to the amounts of protein when the same enzyme preparation was used. An amount of the enzyme which catalysed hydrogen uptake at a rate of 1 μmole per hour was defined as one unit.

Assay of Sulfide and Sulfite Formed—The methods were the same as described in a previous paper (*12*).

Proteins were determined by the Johnson's method (*13*) or by the measurement of optical densities at 280 mμ.

RESULTS

Purification of APS Reductase—A crude enzyme preparation, 96 ml., obtained from 24 g. of wet cells was brought to 0.33 saturation of ammonium sulfate by an addition of the saturated solution, pH of which had been adjusted to approximately 7.0 with ammonium hydroxide. After centrifugation, the supernatant was brought to 0.67 saturation of ammonium sulfate and precipitates were collected and dissolved in 0.05 M phosphate buffer, pH 7.0, and dialysed against distilled water overnight. Two g. of calcium phosphate gel was added to adsorb the enzyme. The elution was carried out with 0.02 M phosphate buffer, pH 7.0, three times and yellow extract was obtained. These extracts were fractionated with ammonium sulfate and precipitates between 0.5 and 0.6 saturation were collected and dissolved in 0.005 M phosphate buffer, pH 7.0. After dialysis against distilled water, this solution was applied on a DEAE-cellulose column, 1.3×9 cm., equilibrated with 0.005 M phosphate buffer, pH 7.0. Chromatography was carried out by a gradient system—the mixing chamber of 300 ml. was filled with 0.005 M phosphate buffer, pH 7.0, and upper chamber was occupied by 0.4 M phosphate buffer, pH 7.0. The active fraction was eluted when phosphate concentration reached 0.06 M. Elution of proteins started at the same concentration. The peak of the enzyme activity coincided with that of protein. However, some tailing was observed in the elution pattern of proteins.

Specific activities of preparations of each step were shown in Table I. When chromatography was repeated twice, the specific activity was raised about 40 times compared with the crude extract.

Properties of the Purified Enzyme—Rate of hydrogen uptake by the purified APS reduc-

TABLE I

Purification of APS Reductase

Step	Protein (mg.)	Specific activity (unit/mg.)	Total activity (unit)
Crude extract	5,570	2.5	13,600
$(NH_4)_2SO_4$ fractionation 1st.	1,660	7.2	12,900
Gel eluate	362	26	9,500
$(NH_4)_2SO_4$ fractionation 2nd	109	45	4,900
DEAE-cellulose chromatography 1st.		63	
DEAE-cellulose chromatography, 2nd.		103	

Unit is μmoles of hydrogen uptake per hour.

TABLE II

Enzymic Activity of APS, Sulfite, Thiosulfate, Sulfate Reduction of Crude Extract and a Purified Preparation

Activity is expressed as μmoles of hydrogen uptake per hour per mg. protein.

Enzyme preparation	Substrate			
	APS	Sulfite	Thiosulfate	Sulfate+ATP
Crude extract	3.9	0.60	2.6	0.47
Purified preparation	75	0.29	0.88	0.0

Activity is expressed as μmol of hydrogen uptake per hour per mg. protein.

TABLE III

Formation of Sulfite in APS Reduction

Procedure is described in METHOD. The enzyme used was gel eluate, 2.0 mg. protein.

APS added μmole	Uptake of hydrogen μmole	Sulfite formed μmole	Sulfide formed μmole
2.75	2.32	1.90	0.0

tase preparation in the presence of the hydrogenase system with APS, sulfite, thiosulfate or sulfate and ATP are shown in Table II.

It was found that the preparation is free from the reductase systems for sulfite and thiosulfate as well as sulfurylase catalysing formation of APS from ATP and sulfate. The results indicate the reductase of APS is different from thiosulfate or sulfite reductase. It was shown in the previous paper (*12*, *14*) that more concentrated phosphate solution was necessary to eluate absorbed thiosulfate reductase or sulfite reductase from calcium phosphate gel. Sulfurylase was also eluted by more concentrated phosphate.*

In APS reduction by the preparation, hydrogen uptake was approximately 1 mole for one mole of APS and accumulation of sulfite was observed in the reaction mixture (Table III).

When the reaction mixture was examined by paper electrophoresis after the hydrogen uptake had ceased, only one ultraviolet-absorbing spot was found, which corresponded to AMP. These results indicate that the APS reduction takes place according to the following equation:

$$APS+H_2=AMP+SO_3^{--}$$

Effects of inhibitors—Effects of various substances on APS reduction are shown in Table IV.

TABLE IV

Effects of Substances on APS Reduction

Substances added	Concentration (*M*)	Inhibition (%)
AMP	10^{-3}	33
	2.5×10^{-3}	55
	5×10^{-3}	67
ICH_2COOH	10^{-3}	85
I_2	10^{-3}	80
$AgNO_3$	10^{-4}	100
$CuSO_4$	10^{-4}	60

ATP 2.5×10^{-3}, ADP 2.5×10^{-3}, GMP 2.5×10^{-3}, UMP 2.5×10^{-3}, CMP 2.5×10^{-3}, Na_2SO_3 2.5×10^{-3}, *p*-Chloromercuribenzoate 5×10^{-4}, $NaAsO_2$ 10^{-3}, KCN 10^{-2}, α, α′-Dipyridyl 10^{-3}, *o*-Phenanthroline 10^{-3}, EDTA 10^{-2}, Quinacrine 10^{-3}, $Na_4P_2O_7$ 10^{-2}, NaF 10^{-3}, $CaCl_2$ 10^{-2}, $BaCl_2$ 10^{-3}, $MgCl_2$ 10^{-3}, Na_2MoO_4 10^{-3}, Na_2SeO_4 10^{-3} had no effect.

As the reaction was measured by hydrogen absorption in the system coupled with hydrogenase, the inhibitory effects might be ascribed to the effect on hydrogenase. It has been, however, proved that all these had only slight effects on the hydrogenase (*15*). Therefore, decrease of the reaction rate can be considered as the inhibition of APS reductase. These results indicate that APS reductase was inhibited by AMP, one of the reaction products, but not by the other product, sulfite. ATP, ADP, GMP, CMP and UMP had no effect at the same concentration. As it was not inhibited by *p*-chloromercuribenzoate or arsenite, this enzyme is not SH enzyme to which sulfite reductase and thiosulfate reductase belong (*14*). NaN_3, KCN and other complex-forming reagents showed no inhibition, so no evidence was obtained whether heavy metals play a role in the enzymic action.

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* unpublished data

Specificity—GPS, UPS and CPS synthesized chemically were tested as electron acceptors instead of APS. Experiments with the purified enzyme preparation showed that the reduction of these substances occurred though somewhat slower than that observed with APS and that hydrogen uptake was almost epual to the molar amount of the nucleotide in every case. (Fig. 1).

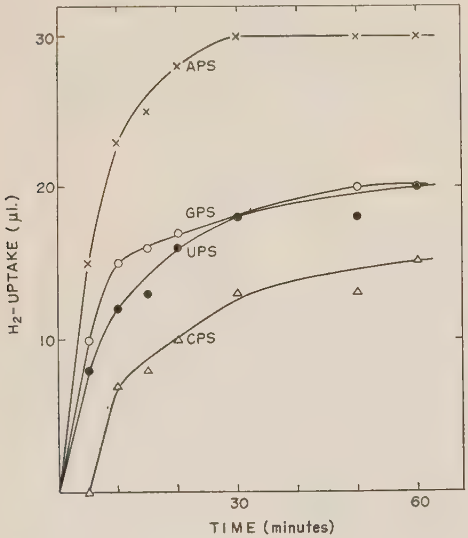

Fig. 1. Reduction of nucleoside phosphosulfates by APS reductase.

Amounts of nucleoside phosphosulfates added were as follows: APS 1.29 μmoles, GPS 0.80 μmoles, UPS 0.92 μmoles, CPS 0.84 μmoles.

As described above, APS reduction was inhibited by AMP but not by GMP, CMP or UMP. On the other hand, reduction of GPS and UPS was inhibited strongly by AMP (86% and 100%, respectively, at $2.5\times10^{-3}\,M$). These facts may indicate the presence of special affinity of AMP and APS to the enzyme. When the crude extract was employed instead of the partially purified enzyme, GPS, CPS and UPS were reduced with consumption of 4 moles of hydrogen forming approximately 1 mole of sulfide. With crude extract the rate of reduction was also somewhat lower than that of APS reduction as in the purified enzyme system. These results suggest that APS reductase also acts on the other nucleoside phosphosulfates and there is no evidence supporting the presence of other enzymes in the reduction of each nucleoside phosphosulfates.

Reversibility—The reversed reaction by this enzyme, that is, oxidation of sulfite in the presence of AMP to APS:

$$SO_3^{--}+AMP=APS+2H$$

was tested by means of S^{35}-sulfite. A solution containing 0.5 ml. of purified APS reductase, 5 μmoles of AMP, 10 μmoles of S^{35}-K_2SO_3 (34,000 c. p. m.), 50 μmoles of phosphate buffer, pH 7.0, 0.02 μmoles of cytochrome c_3 was incubated in air for 1 hour at 37°C. Reaction was stopped by heating the solution in a boiling water bath for 90 seconds and 12 μmoles of $BaCl_2$ was added in order to eliminate a major part of S^{35}-SO_3^{--} and S^{35}-SO_4^{--} formed during incubation, then the supernatant diluted with 100 μmoles of non-radioactive sulfate and sulfite. After centrifugation, nucleotides in the supernatant were adsorbed by Norit (75 mg.), which was washed with 250 ml. of water, and eluted with 20 ml. of ammoniacal ethanol. After APS was added as a carrier, the eluate was evaporated in vacuum and the concentrate was examined by paper electrophoresis (ammonium acetate buffer, pH 5.5, 3.5 hours, 10 volts/cm.) and radioautography. Under ultraviolet light two spots were detected. One was due to AMP and the other due to APS which corresponded exactly to the radioactive spot.

This results indicated the formation of APS. When either enzyme or AMP was omitted, or when S^{35}-SO_4^{--} was substituted for S^{35}-SO_3^{--}, no radioactive spot was found. These facts suggest that APS reductase can catalyze the reverse reaction. In this case cytochrome c_3 was added as an intermediary carrier transferring electron to oxygen, a terminal electron acceptor. But curiously, omission of cytochrome c_3 gave little effect on the APS formation. It has not been elucidated whether a slight amount of cytochrome c_3 in the APS reductase preparation played a role as a carrier or the APS reductase or other electron carrier systems linked to it, is autoxidizable.

DISCUSSION

In these experiments reported above, methylviologen was employed exclusively as the hydrogen donor for the APS reduction. Among natural pigments, cytochrome c_3 was also utilizable (*16*), but TPNH, FAD or dihydrolipoate which is an effective hydrogen donor in PAPS reduction were not utilized (*17*). The facts that the reduction velocity and concentration of the enzyme were paralel and that a purification of forty times was attained with considerably good yield gave support to the participation of a single enzyme in APS reduction. The partially purified preparation obtained in DEAE-cellulose column chromatography was unstable and further purification was abandoned.

The specificity of the reductase is rather wide; in contrast, the specificity of sulfurylase is restricted to APS. The results that the latter enzyme did not act on GPS and UPS at all and on CPS only a little were obtained by the experiments on the nucleoside triphosphate formation from the nucleoside phosphosufate and pyrophosphate in the extract of *D. desulfuricans**. Wilson and Bandurski found that GTP and UTP were not substrates of sulfurylase of yeast in an experiment on pyrophosphate formation in the presence of molybdate. They also found that CTP took part in the reaction only weakly. Accordingly GPS, UPS and CPS reduction may have no significance in the sulfate reduction. As the reductase did not act on PAPS (*4*, *6*) and had special affinity to AMP, it is better to call it APS reductase rather than nucleoside phosphosulfate reductase. So as to elucidate the specificity of the enzyme, other phosphosulfate compounds have to be tested.

Three mechanisms may be considered for the reduction of APS by the enzyme. Those were 1) direct reduction of APS to AMP and sulfite, 2) reduction of APS to adenosine phosphosulfite, which is then hydrolysed to to AMP and sulfite and 3) transfer of sulfate group to some other group and then reduction of formed sulfuryl compound to sulfite. The first possibility is somewhat analogous to the reductive fission of phosphate anhydrides in the case of 3-phosphoglyceraldehyde dehydrogenase reaction. Adenosine phosphosulfite has not yet been obtained and it is considered to be a very unstable compoud (*18*). The possibility of the intermediary formation of S-sulfonate was proposed by Hilz *et al.* (*19*) for the PAPS reduction in yeast. In their experiments, SH-groups were necessary for the reaction. S-sulfonate is known to react easily with sulfhydryl compounds to give sulfite, but the participation of sulfhydryl groups is excluded in APS reduction by the fact that SH reagents showed no inhibition.

It was stated that APS is an intermediate in sulfate reduction of *D. desulfuricans*, while PAPS is an intermediate in that of yeast and *E. coli*. Formation of APS was achieved in consumption of one molecule of ATP, while PAPS formation required another molecule of ATP in addition. On the other hand, PAPS can accumulate more easily than APS according to the results on liver sulfate-activating system (*20*). It is interesting that APS reduction participates in the sulfate respiration which should supply energy and PAPS reduction participates in the sulfate assimilation. The fact that activity of the APS reductase in *Desulfovibrio* is about 1,000 times higher than that of PAPS reductase in yeast and *E. coli*, indicates also the difference of physiological significance of sulfate reduction in both types of organisms.

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* unpublished data

SUMMARY

Adenosine-5′-phosphosulfate reductase was purified by 40 times from the extract of *Desulfovibrio desulfricans.* This preparation catalyzed the reduction of APS to sulfite using methylviologen as an electron carrier. It can also reduce guanosine-5′-phosphosulfate, cytidine-5′-phosphosulfate and uridine-5′-phosphosulfate. AMP inhibited the APS reduction but SH reagent such as arsenite or *p*-chloromercuribenzoate or metal chelating agents had no effect. Reversibility of APS reductase was also confirmed.

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Studies on Laccases of Lacquer Trees

II. Copper Exchange Studies of *Rhus vernicifera* Laccase Using Radioactive Copper

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The presence of copper in laccase was first discovered by Keilin and Mann (*1*, *2*) who purified a laccase from latex of *Rhus succedanea* and found that the purified preparation contained 0.24% copper. A few years later, Yakushiji (*3*) purified a laccase from a mushroom (*Lactarius piperatus*) and showed that the purified enzyme also contained copper. Tissières' work (*4*) on the reconstruction of laccase from its protein moiety and copper verified clearly the essential role played by copper in the enzymatic activity. Recent studies made by Nakamura (*5*) showed that the copper atom in laccase underwent reversible changes between cupric and cuprous forms; it was reduced to the cuprous form in the presence of a suitable substrate, and oxidized to cupric by oxygen. But the precise state of copper in laccase is still obscure.

In the preceding paper (*6*), the properties of *Rhus vernicifera* and *Rhus succedanea* laccases were described and compared. This paper deals with the studies on the state of copper in *R. vernicifera* laccase using radioactive copper. *R. vernicifera* laccase was preferred to *R. succedanea* laccase since the purification of the former was easier, and the yield of the purified enzyme was also higher than the latter.

EXPERIMENTAL

Preparation of R. vernicifera Laccase—The purification procedure of *R. vernicifera* laccase was described in the preceding report (*6*). All the enzyme preparations used in this study were electrophoretically pure.

Radioactive Copper—Radioactive copper (Cu^{64}) was manufactured by irradiating pure copper with neutrons in the JRR-1 reactor of the Japan Atomic Energy Research Institute. The irradiated copper was dissolved in nitric acid, and excess nitric acid was expelled by evaporation. Radioactive cupric nitrate thus prepared was dissolved in water, and used in the following experiments. Specific radioactivity of the copper ranged from 2.0 to 0.5 milli-curies per g.

When the radioactive copper solution was extracted with carbon tetrachloride solution of dithizone, all of the radioactivity was extracted in the carbon tetrachloride layer. Half-life measurements of beta- and gamma-radiations over a week revealed no radioactive impurities.

Counting Procedure—The radioactivity of laccase was counted as follows. The enzyme solution was wet-ashed to colorless by heating with 2 ml. of concentrated sulfuric acid. The digested solution was diluted to 10 ml. with water, neutralized to pH 2.0 with 4 N aqueous ammonia. Ten ml. of 0.1 M citrate buffer (pH 2.0) was added to the solution, and the mixture was extracted with 5 ml. of 0.02% dithiozone in carbon tetrachloride by shaking in a 100 ml. separating funnel. If the green tint of dithiozone disappeared completely, the extraction procedure was repeated by adding more dithizone solution. The extracted copper dithizonate solutions were combined, and concentrated to a few ml. by evaporating under a 300 watt floodlamp. The concentrated extract was then evaporated to dryness by dripping on a heated aluminum counting dish with a small disk of filter paper (1 cm. in diameter) placed on its center, and its radioactivity was measured by an end window-type, lead-shielded counter. One sample was usually counted for 30 minutes, and all counts were corrected for background and decay. The background was 450 to 500 counts per 30 minutes.

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Separation of Ionic Copper from Laccase—An ion-exchange resin treatment similar to that employed by Joselow and Dawson (*7*) in their study on ascorbic acid oxidase was used. After incubating laccase and radioactive copper together, the ionic copper was removed by passing the solution through a small column (2 cm. in diameter ×4 cm. high) of Amberlite XE-64 resin bufferized with 0.1 *M* acetate buffer of pH 6.0. *R. vernicifera* laccase was not adsorbed on the resin at these ionic strength and pH, while ionic copper was completely adsorbed and removed. By washing the column with 30 ml. of 0.2 *M* acetate buffer of pH 6.0, more than 95 per cent of laccase was constantly recovered in the effluent. The column was gently suctioned by an aspirator, and it took about 5 minutes to treat one sample solution in this manner.

Other Procedures—Copper determination and laccase activity measurements were performed as described in the preceding paper (*6*).

RESULTS

Preliminary Experiments Using Non-Radioactive Copper—Some preliminary experiments were carried out to determine whether the copper contents of *R. vernicifera* laccase increased or not by the contact with ionic copper. Laccase solutions were incubated with large amounts of ionic copper for 30 minutes under various conditions as described below, and then the solutions were passed through the resin column to remove ionic copper. Copper contents and laccase activity of the effluent were measured. During incubation, temperature was kept at 25°C.

The standard reaction mixture consisted of 10 ml. of 0.1 *M* acetate buffer of pH 6.0, 1 ml. of *R. vernicifera* laccase solution, and 1 ml. of cupric nitrate solution.

Exp. 1:	Standard reaction mixture.
Exp. 2:	1 ml. of 0.01 *M* hydroquinone solution was added to the standard reaction mixture, and the solution was shaken during incubation period.
Exp. 3:	0.2 g. of sodium chloride and 1 ml. of 0.01 *M* sodium ascorbate solution were added to the standard reaction mixture, and the mixture was incubated anaerobically.
Exp. Std.:	Cupric nitrate solution was omitted from the standard reaction mixture.

The results obtained are summarized in Table I. From the results it was concluded that 1) laccase passed through the column without loss of activity and copper (Exp. Std), 2) its copper content was not increased by the contact of oxidized enzyme with cupric ion (Exp. 1), reduced enzyme with cuprous ion (Exp. 3), or by the enzymatic oxidation of hydroquinone in the presence of ionic copper (Exp. 2). Therefore, if any radioactivity is found to be incorporated into laccase by incubating the enzyme with radioactive copper, the incorporation can surely be regarded as due to an exchange reaction.

Oxidized Laccase and Cupric Ion—Ten ml. of 0.1 *M* acetate buffer of pH 6.0, 1 ml. of *R. vernicifera* laccase solution, and 1 ml. of radioactive cupric nitrate solution were mixed and the mixture was kept at 25°C for varying time-lengths, and then ionic copper was removed by passing the reaction mixture through the resin column. The effluent was

TABLE I

Incubation of Laccase with Ionic Copper

Exp. No.	Laccase added to the reaction mixture			Cupric nitrate added	Laccase recovered in the effluent	
	dry wt. mg.	total activity µl. O_2/hr.	Cu µg.	Cu µg.	total activity µl. O_2/hr.	Cu µg.
Std	21.7	1.35×10^5	55.8	0	1.40×10^5	54.0
1	〃	〃	〃	890	1.54× 〃	55.0
2	〃	〃	〃	〃	1.43× 〃	54.2
3	〃	〃	〃	〃	1.53× 〃	56.0

divided into two parts, one of which was rapidly concentrated and digested with sulfulic acid for the determination of radioactivity, and the other was left for several days until the radioactivity of copper decayed to a very low level, and then the copper contents was determined by dithizone method. Radioactivity and copper contents measured were converted to the value corresponding to the total effluent, and recorded in Table II.

It was found that small amounts of radioactivity was rapidly incorporated into laccase by the contact of oxidized enzyme with radioactive cupric ion. But this incorporation of radioactivity was not due to a true exchange reaction between laccase copper and ionic copper, for the incorporation of radioactivity did not increase with incubation time, and, moreover, the incorporated radioactivity was found to be completely lost when the labelled laccase was incubated with large amounts of non-radioactive cupric nitrate for 60 minutes. These results may be understood by assuming that purified laccase has the capacity of combining small amounts of excess cupric ion, the combination of which is far weaker than that of essential copper with laccase-protein, yet strong enough to hold the excess copper through the resin treatment.

Table II

Copper Exchange between Oxidized Laccase and Cupric Ion

Exp. No.	Laccase added		Cupric nitrate added	Incubation time	Laccase recovered		Comparison standard of radioactivity	Exchange
	dry wt. mg.	Cu μg.	Cu μg.	min.	Cu μg.	radioactivity counts/30min.	counts/30 min.[1]	per cent
1	22.0	56.5	1,570	5	56.0	949±36	29,310±170	3.2
2	〃	〃	〃	20	57.2	1,122±39	〃	3.8
3	〃	〃	〃	40	55.8	1,278±41	〃	4.4
4	〃	〃	〃	60	55.0	1,160±40	〃	4.0
5	27.6	76.0	3,720	20	76.5	131±23	3,740±62	3.8
6	〃	〃	〃	60	76.2	103±22	〃	3.0

1) This value is the radioactivity of laccase copper when 100% exchange of copper between laccase and ionic copper is attained.

$$\text{Total radioactivity added} \times \frac{\text{laccase-Cu}}{\text{laccase-Cu} + \text{cupric nitrate-Cu}}$$

If this was the case, the copper contents of laccase should have been increased by the incorporation of the excess copper. But the increment was so small that in the preliminary experiments using non-radioactive copper it was not possible to detect such incorporation. It should be pointed out that the copper analysis by the dithizone method is usually accompanied by an experimental error of about 2 per cent. The nature of this excess copper is unknown, but, as its amounts is very small and its combination has no effect upon the activity of the enzyme, its existence may be neglected and it may be safely concluded that the essential copper of oxidized laccase does not exchange with cupric ion in the medium.

Reduced Laccase and Cuprous Ion—Ten ml. of 0.1 *M* acetate buffer of pH 6.0 containing 0.2 g. of sodium chloride, 1 ml. of laccase solution, 1 ml. of radioactive cupric nitrate solution, and 1 ml. of 0.01 *M* sodium ascorbate solution were mixed and incubated anaerobically at 25°C. Laccasse and ionic copper were reduced rapidly and kept reduced during the incubation period. After incubation, ionic copper was removed, and the copper contents and the radioactivity of laccase were measured (Table III).

The copper of reduced laccase exchang-

Table III

Copper Exchange between Reduced Laccase and Cuprous Ion

Exp. No.	Laccase added		Cupric nitrate added	Incubation time	Laccase recovered		Comparison standard of radioactivity	Exchange
	dry wt. mg.	Cu µg.	Cu µg.	min.	Cu µg.	radioactivity counts/30 min.	counts/30 min.	per cent
1	27.6	76.0	485	20	76.8	7,882±91	9,150±98	86.4
2	//	//	//	40	76.2	8,435±94	//	92.5

Table IV

Copper Exchange during the Enzymatic Oxidation of Hydroquinone

Exp. No.	Laccase added		Cupric nitrate added	Cocentration of hydroquinone solution	Laccase recovered		Comparison standard of radioactivity	Exchange
	dry wt. mg.	Cu µg.	Cu µg.	*M*	Cu µg.	radioactivity counts/30min.	counts/30 min.	per cent
1	22.0	56.5	1,570	0	—	875±36	22,800±170	3.8
2	//	//	//	0.010	—	1,923±48	//	8.5
3	//	//	//	0.020	—	3,528±63	//	15.5
4	27.1	67.7	917	0	67.0	604±32	18,120±136	3.3
5	//	//	//	0.010	66.8	1,274±41	//	7.0

ed with cuprous copper, and the exchange was almost complete after the incubation of 20 minutes. The incorporated radioactivity was not driven out by the contact of the labelled laccase in oxidized state with large amounts of non-radioactive cupric ion for 60 minutes.

Copper Exchange during Oxidation of Hydroquinone—The possibility of an exchange reaction occurring between the copper of laccase and ionic copper during the enzyme reaction was investigated. Ten ml. of 0.1 *M* acetate buffer of pH 6.0, 1 ml. of laccase solution, 1 ml. of radioactive cupric nitrate solution. and 1 ml. of hydroquinone solution of varying concentrations were mixed together, and the mixture was shaken for 30 minutes at 25°C. During incubation, hydroquinone was comletely oxidized; the oxidation was almost complete in the initial 10 minutes. The relation between the incorporated radioactivity and the amounts of hydroquinone added is shown in Table IV.

The copper of laccase was expected to exchange with ionic copper while the enzyme was catalyzing the oxidation of the substrate, as the copper of reduced laccase exchanged with cuprous ion, and as the laccase copper was known to undergo reversible changes between cupric and cuprous forms during the catalysis (*5*). The results obtained were somewhat contrary to expectation. The laccase copper exchanged with ionic copper, and the extent of exchange increased with the amounts of added hydroquinone, which had been oxidized by the catalysis of laccase, but the degree of exchange was rather low.

The oxidation-reduction potential of cupric ion-cuprous ion system is lower than that of hydroquinone-*p*-benzoquinone system. Therefore, almost all of the added cupric ion in the reaction mixture must have been in cupric state. This situation suggested the necessity of the following experiment on the copper exchange between reduced laccase and cupric ion.

Reduced Laccase and Cupric Ion—The oxidation-reduction potential of laccase (+415 mV. at 25°C, pH 7.0) (*8*) is higher than that of hydroquinone-*p*-benzoquinone system (+270 mV. at pH 7.0) (*9*), and the enzyme can be completely reduced by the addition of hydroquinone, while cupric ion-cuprous ion system has a much lower oxidation-reduction potential (+170 mV. at 25°C, pH 0.0) (*10*). When a small amount of hydroquinone was added to the mixed solution of laccase and cupric nitrate in acetate buffer under anaerobic conditions, laccase was rapidly reduced and kept in the reduced state; the ionic copper, however, was not reduced, and the optical density at 750mμ was not perceptibly changed. Exchange reaction between laccase copper and ionic copper under these conditions was studied.

Ten ml. of 0.1 *M* acetate buffer of pH 6.0, 1 ml. of laccase solution, 1 ml. of radioactive cupric nitrate solution, and 0.3 ml. of 0.01 *M* hydroquinone solution were mixed and incubated anaerobically for varying timelengths at 25°C. After incubation, ionic copper was removed, and the copper content and the radioactivity of laccase were measured (Table V).

Some exchange took place, but the exchange reaction was not dependent on incubation time, and the degree of exchange was low. The incorporated radioactivity was not expelled by the contact with non-radioactive cupric nitrate. The interpretation of these results is rather difficult. If the incorporation of radioactivity takes place through reduced laccase-cupric ion exchange reaction, namely the replacement of the cuprous copper atom of reduced laccase by cupric ion, the incorporated radioactivity must increase with incubation time. If it proceeds by the exchange reaction between reduced laccase and cuprous ion, very small amounts of which must be present in the reaction mixture in an equilibrium with cupric ion in the presence of hydroquinone, the exchange should go to completion, since the work by Nazarenko and Kriss (*11*) showed that the isotope exchange between cuprous and cupric ions is very rapid, and it goes to completion regardless of the presence or absence of oxygen. More experiments using varying amounts of hydroquinone and cupric nitrate are needed to elucidate the nature of this exchange reaction. But, at present, it may be safely concluded that the exchange reaction of copper atom between reduced laccase and cupric ion does not take place, or at least, is a very slow reaction when compared with the rapid exchange reaction of copper atom between reduced laccase and cuprous copper.

DISCUSSION

The data reported in this paper indicate that the copper of reduced laccase rapidly exchanges with exogenous cuprous ion, but no exchange takes place between oxidized laccase and curpic ion. These findings suggest that the copper of oxidized laccase is much more firmly bound to the protein moiety than that of reduced laccase. This suggestion seems to be supported by the study of Duffield and Calvin (*12*) who showed that the rate of copper exchange in pyridine solution between cupric acetate and various copper chelate compounds is strictly dependent on the stability of the chelate compounds.

It was also found in the present study

Table V

Copper Exchange between Reduced Laccase and Cupric Ion

Exp. No.	Laccase added		Cupric nitrate added	Incubation time	Laccase recovered		Comparison standard of radioactivity	Exchange
	dry wt. mg.	Cu μg.	Cu μg.	min.	Cu μg.	radioactivity counts/30 min.	counts/30 min.	per cent
1	27.6	76.0	485	10	77.7	1,339±41	15,100±159	8.9
2	//	//	//	20	77.9	1,370±42	//	9.1
3	//	//	//	40	76.8	1,648±45	//	10.8

that some exchange of copper can be observed when laccase is catalyzing the oxidation of hydroquinone in the presence of copper ion. The velocity of exchange in this case is, however, much slower than that of the rapid exchange between reduced laccase and cuprous ion. This is in sharp contrast to the results obtained with ascorbic acid oxidase, the copper of which has been shown to exchange with exogenous copper ions only when the enzyme is actively catalyzing the oxidation of its substrate (*13*). Therefore, it appears very likely that the state of copper in laccase is quite different from that in ascorbic acid oxidase, although these two enzymes show similar absorption spectra and catalyze the oxidation of the same substrate, *i.e.* L-ascorbic acid. That the state of copper is different in the two enzymes may also be inferred from the observed differences in their kinetics: ascorbic acid oxidase is gradually inactivated during its catalysis of L-ascorbic acid oxidation and this inactivation is markedly increased by the presence of cupric ion (*14*), while no such inactivation can be observed with laccase when L-ascorbic acid is used as the substrate (*6*) and the addition of curpic ion does not affect the reaction kinetics.

Owing to the high oxidation-reduction potential of laccase, it was further possible in the present study to investigate the exchange of copper between reduced laccase and cupric ion. Although more experiments are still needed to draw any decisive conclusions, the results obtained seem to suggest that cupric ion is unable to exchange with the cuprous copper of the reduced enzyme. This finding is of interest in view of the fact that the copper of laccase can take both cupric and cuprous forms and in its cuprous form can freely exchange with exogenous cuprous ion.

The data reported by Joselow and Dawson (*15*) seem to be pertinent in this connection. These workers showed that the cuprous copper of deoxygenated hemocyanin is also unable to exchange with cupric ion added in the medium and interpreted the finding as supporting the assumed high affinity of hemocyanin-copper to the protein moiety. However, the possibility is still to be considered that cuprous ion (but not cupric ion) is much more readily exchangeable with the cuprous copper of deoxygenated hemocyanin as is the case of laccase.

Finally, it should be recalled that all the experiments in the present study were carried out in 0.1 *M* acetate buffer of pH 6.0 in which most of the cupric ion added exists in the form of a cupric acetate complex. The actual concentration of free cupric ion should, therefore, be much lower than that calculated from the amount of added cupric nitrate. The complex is, however, not very stable (dissociation constant is of the order of 10^{-3} (*16*)) and its existence in the reaction mixture does not seem to interfere with at least the qualitative reliability of the exchange experiments. When the exchange between cuprous ion and reduced laccase was measured, an excess of sodium chloride was also added to the reaction mixture to stabilize the cuprous ion. Since cuprous ion forms a stable complex with chloride under these conditions, the concentration of free cuprous ion in the medium must again far lower than in the absence of sodium chloride. At present it is not possible to decide which of the two cuprous components, free cuprous ion and cuprous chloride complex, is actually responsible for the rapid replacement of the cuprous copper atom of reduced laccase.

In spite of these complications, however, it seems plausible to draw the following conclusions based on the results described in the present paper: 1) the copper-protein linkage in reduced laccase is much weaker than that in oxidized laccase; 2) the cuprous copper of the reduced enzyme can readily dissociable from and re-enter into the laccase molecule; and 3) the cupric copper of the oxidized enzyme shows no tendency to dissociate from the protein, nor can exogenous cupric ion enter into the molecule to replace the labile cuprous copper.

SUMMARY

1. The exchange reaction of laccase copper with ionic copper under varying con-

ditions was studied using radioactive copper as the tracer.

2. The copper atom of oxidized laccase did not exchange with cupric ion in the medium. The copper-protein linkage of oxidized laccase must be very stable.

3. The copper atom of reduced laccase exchanged with cuprous ion rapidly, but it can not be replaced by the cupric ion in the medium.

4. The copper atom of laccase exchanged with ionic copper while the enzyme was catalyzing the oxidation of hydroquinone, and the extent of exchange increased with the amounts of hydroquinone oxidized, but the velocity of exchange was very slow compared with the rapid exchange reaction between reduced laccase and cuprous ion.

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Chemical Activities of *Azotobacter agile* Adsorbed on a Resin

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As reported previously (*1–3*), cells of *E. coli* oxidize some organic materials more slowly when the cells are adsorbed on a resin, Dowex-1, (adsorbed cells) than when they are suspended in a solution (free cells). This phenomenon was defind to be 'A effect' (*3*). In each case of oxidation the form of activity-pH curve with adsorbed cells is similar to that with free cells, while the optimum pH of the former is higher (about one pH unit) than that of the latter ('B effect'). It is of interest to examine whether 'A and B effect' will be observed in chemical activities of other bacteria, when the cells are adsorbed on the resin. It is also of importance to investigate further other effects, which might be observed in chemical activities of bacteria adsorbed on the resin. The present report is concerned with chemical activities of *Azotobacter agile* adsorbed on the resin.

MATERIALS AND METHODS

Organism—Azotobacter agile ATCC 9046 (*4*) was grown at 30°C by shaking in a medium composed of Starkey's salt solution (*5*) with the addition of 1% glucose or of 0.5% succinate. Duration of cultivation was 24 hours and 12 hours, when glucose and succinate, respectively, were used as sole source of carbon. The cells were harvested by centrifugation (4000 r.p.m. for 15 minutes) and, after being washed with distilled watr, suspended in the same medium (free cells). The number of cells was counted by the plate method. The adsorbed and desorbed cells were then prepared as described in the previous paper (*3*).

Measurement of Activity—All the procedures employed were as described in the previous report (*3*).

Spectrophotometric Analysis—Spectrophotometric analysis was carried out with a Beckman spectrophotometer Model DU.

RESULT AND DISCUSSION

A Effect—When cells were grown in the medium containing glucose as a sole carbon source (glucose-grown cells), activity of *M*/100 glucose oxidation with adsorbed cell (at pH 7.0) was observed to be about 26 per cent of that with free cell. When cells were grown in the medium containing succinate (succinate-grown cells), rate of *M*/100 succinate oxidation with adsorbed cells (at pH 7.0) was about 34 per cent of that with free cells. It is, therefore, concluded that 'A effect' is common to *E. coli* and *A. agile.*

B Effect—In the case of glucose-grown cells, the shape of activity-pH curve with adsorbed cells was similar to that with free cells, and the optimum pH of the former was higher by about one pH unit than that of the latter, as shown in Fig. 1a. When cells were grown in the medium containing succinate, the shape of activity-pH curve of succinate oxidation with adsorbed cells was not similar to that with free cells, though the optimum pH of adsorbed cells was higher by about one pH unit than that of free cells (Fig. 1b). However, taking into account of the activity-pH curve with desorbed cells of succinate grown cells (Fig. 1 b), the difference in the form between two activity-pH curves, with free and adsorbed cells, will be interpreted in terms of the following considerations as explained in the case of succinate oxidation with adsorbed cells of *E. coli* (*3*); when the free cells were adsorbed on the resin some materials in the cells, involved specifically in the succinate oxidation, would be pulled out into the resin or inactivated, resulting in the transfiguration of the activity-pH curve to that of desorbed cell. And by the same cause for 'B effect', the transfigured curve would be moved to alkaline side by about one pH unit and would be coincide with that of adsorbed cells.

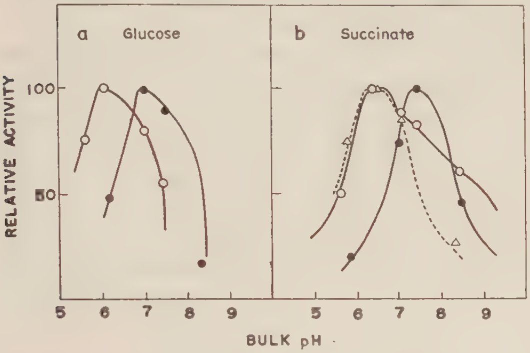

FIG. 1. Activities of glucose and succinate oxidation with free and adsorbed cells as a function of pH. Open and closed circles indicate activity of oxidation by free and adsorbed cells, respectively. Dotted line shows oxidation by desorbed cells (△).

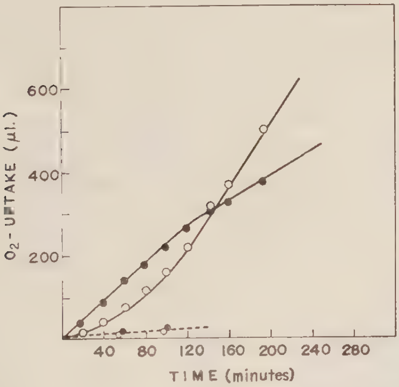

FIG. 2. $M/10$ succinate oxidation with free and adsorbed cells at pH 7.0. Open circles indicate oxidation with free cells (1.2×10^8 cells), and closed circles with absorbed cells (3.0×10^8 cells). Dotted line indicates endogeneous respiration for each cell.

These results indicate that 'A and B effect', observed in chemical activities of *E. coli*, were also observed in chemical activities of *A. agile* when the cells were adsorbed on the resin, and these effect may be interpreted as in the case of *E. coli* (*3*).

Induced Oxidation of Succinate—It should be noted that the lag time observed in succinate oxidation with free, glucose-grown cells disappeared, when the cells were adsorbed on the resin, as shown in Fig. 2.

Suto (*4*) reported that the appearence of the lag time in the succinate oxidation with *A. agile* was the reflection of the induced formation of a succinate-permeating system. In the case of induced oxidation of lactose or xylose with *E. coli*, the lag time observed with adsorbed cells was shorter than that with free cells, as reported previously (*1*). Such effects of the adsorption of cells on to the resin on induced processes will be called 'C effect'.

With desorbed, glucose-grown cells the lag time being observed in succinate oxidation also disappeared as shown in Fig. 3, or it was apparently shorter than that with free cells even in the case when the lag time appeared. From the result, it was supposed that in 'C effect' some irreversible processes were involved. To analyze the 'C effect', next experiments were carried out. Free, glucose-grown cells were suspended in water (10^{12} cells/ml.), heated in a boiling water bath, and then centrifuged to obtain a supernatant (4,000 r. p. m. for 15 minutes). When

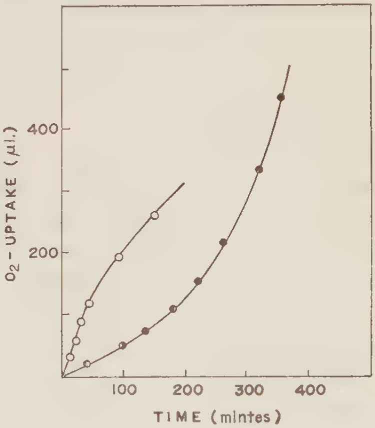

FIG. 3. $M/10$ succinate oxidation at pH 7.0 with desorbed cells grown in presence of glucose. Open and closed circles indicate activity of oxidation by desorbed cells (about 3×10^8) without and with hot-water extract of free, glucose-grown cells, respectively.

0.1 ml. of the extract thus prepared was added to the reaction mixture containing desorbed cells, the lag time became conspiciously long as shown in Fig. 3. However, the hot-water extract from succinate-grown cells did not exhibit such effect on the lag time of succinate oxidation with desorbed, glucose-grown cells.

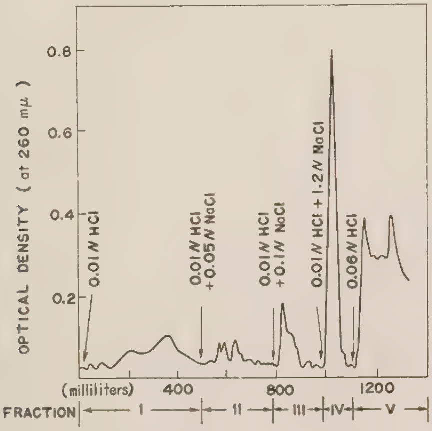

Fig. 4. Separation of effective component of hot-water extract of free, glucose-grown cells. Colum: Dowex-1, chloride form, 100–200 mesh 15 cm. × 0.5 cm^2. Adsorbed material: hot-water extract from about 5×10^{13} cells.

The hot-water extract from glucose-grown cells was hydrolysed with N HCl solution in a boiling water bath for 1 hour or with 0.3 N KOH solution at 37°C for 18 hours and, after neutralization, was tested of the effect on desorbed, glucose-grown cell. Acid and alkaline hydrolysates of the extract exhibited no effect on the succinate oxidation with the desorbed cells. To separate the effective component of the extract, it was passed through the column of the same resin as used in the preparation of adsorbed cells. It was found that the component was completely adsorbed on the resin. After the column was treated with 0.004 N HCl solution, the elution of the component was performed using 0.01 N HCl solution with increasing concentrations of NaCl (Fig. 4). The effective component was thus found to be present only in the fraction IV. Other fractions showed no effect on the lag time, and the fraction I rather enhanced the rate of succinate oxidation with desorbed cell (Table I). Absorption spectrum of the fraction IV showed a maximum at 260 mμ as shown in Fig. 5. The effective component, therefore, was supposed to be nucleotides. In reference to the results of Volkin and Cohn (*6*), nucleotides in the fraction IV might be assumed being composed of hexa- or heptanucleotides.

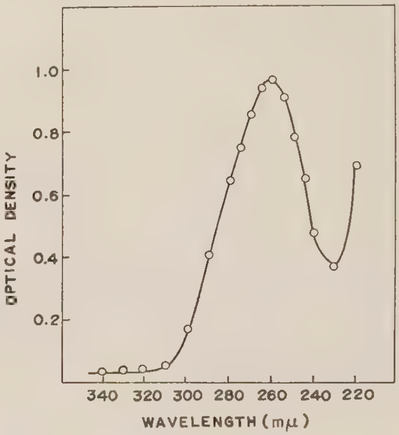

Fig. 5. Ultraviolet absorption spectrum of the fraction IV.

Table I

Effect of the Fractions in Fig. 4 on the Lag Time of Succinate Oxidation with Desorbed Cells.

	added fraction[1]	relative value of lag time[2]
free cell	none	1
desorbed cell	none	<0.8
desorbed cell	I	<0.8
desorbed cell	II	<0.8
desorbed cell	III	<0.8
desorbed cell	IV	>3.5[3]
desorbed cell	V	<0.8

1) Each fraction was prepared to show 0.1 O.D. unit of optical dencity at 260 mμ, and 0.1 ml. of each aliquot was added to 2 ml. of the reaction mixture.

2) Duration of lag time of succinate oxidation with free cells (70—90 minutes) was arbitarily taken as one.

3) In this case duration of lag time was more than 250 minutes.

From these results, 'C effect' observed in succinate oxidation with glucose-grown cells will be interpreted as follows: some materials, probably uncleotides, may be involved in the control machanism of induced formation of a succinate permeating system, and they may be pulled out from the cells, when the cells were adsorbed on the resin. It might also be supposed that these materials would play a role in blocking the succinate permeating system in glucose-grown cells. The induced formation of the succinate permeating system would be resulted from the process of elimination (or transformation) of the materials by the action of an inducer such as succinate.

SUMMARY

1) Cells of *A. agil* were adsorbed on a resin, Dowex-1 of the chloride form (adsorbed cells). As in the case of *E. coli*, adsorbed cells of *A. agile* oxidized glucose or succinate more slowly than the cells suspended freely in a solution ('A effect'). In the case of glucose oxidations activity-pH curve with adsorbed cells was similar to that with free cells, and pH value for a maximum of the former was higher by one unit than that of the latter ('B effect').

2) The lag time, observed in induced oxidation of succinate with free, glucose-grown cells, disappeared when the cells were adsorbed on the resin ('C effect'). In succinate oxidation with desorbed, glucose-grown cells the lag time also disappeared. A nucleotides fraction obtained from hot-water extract of glucose-grown cells showed an effect on the lag time of desorbed cells to prolong conspiciously, but the same extract obtained from succinate-grown cells did not exhibit such an effect. Possible mechanism involved in the 'C effect' was disccussed.

Authors thanks are due to Prof. T. Uemura and Dr. T. Suto for their valuable suggestions, and to Miss T. Konno for her skilful technical assistance.

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Microbiological and Enzymatic Studies on the Isoleucine Isomers

I. Utilization of the Stereoisomers of Isoleucine by Lactic Acid Bacteria

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Although there have been several reports concerning the microbiological activity of the four stereoisomers of isoleucine for the growth of lactic acid bacteria (*1-4*), a definite conclusion has not been drawn from these studies. Especially in the previous studies on the utilization of isoleucine isomers by *Lactobacillus arabinosus* 17-5, there have been considerable discrepancies between the results obtained by earlier investigators. For instance, Hegsted (*1*) reported that L-*allo*isoleucine has 25-50 per cent of the activity of L-isoleucine for the growth of *Lact. arabinosus* 17-5, and Meister (*3*) concluded that L-*allo*isoleucine has approximately the same activity as L-isoleucine for the growth of this organism. It was also reported by these investigators that D-isoleucine and D-*allo*isoleucine show no activity. On the other hand, Koser *et al.* (*4*) showed that *Lact. arabinosus* 17-5 is able to utilize D-isoleucine partially.

In the present report, the microbiological activities of the four stereoisomers of isoleucine for lactic acid bacteria such as *Streptococcus faecalis* R, *Leuconostoc mesenteroides* P-60 and *Lactobacillus arabinosus* 17-5, which are widely used for microbioassay of amino acids, are compared both in the presence and absence of vitamin B_6 compounds. As a result, several interesting facts are discovered that *Str. faecalis* R and *Leuc. mesenteroides* P-60 are able to utilize only L-isoleucine in either presence or absence of vitamin B_6 compounds; on the other hand, *Lact. arabinosus* 17-5, in contrast with the earlier reports, is able to utilize not only L-isoleucine and L-*allo*isoleucine but also D-isoleucine and D-*allo*isoleucine which have been reported to be not utilized by this microorganism, in the presence of vitamin B_6 compound. It was also found that this strain can not utilize L-*allo*isoleucine, D-isoleucine and D-*allo*isoleucine in the absence of vitamin B_6 compounds.

The results obtained with *Lact. arabinosus* 17-5 are of interest and are quite different from the earlier reports (*1-4*). Therefore, further experiments were designed to make clear these discrepancy and the extensive investigations on the utilization of isoleucine isomers by *Lact. arabinosus* 17-5 were studied in detail.

EXPERIMENTAL

Stereoisomers of Isoleucine—The four isomers of isoleucine were prepared by the enzymatic resolution described by Greenstein (*5, 6*). The commercially available samples of L-isoleucine, D-isoleucine (from General Biochemicals, Inc.) and D-*allo*isoleucine (from Mann Research Laboratories, Inc.) were also used, and gave the same results as that observed with the sample prepared by the author.

Test Organisms—Three strains, *Str. faecalis* R, *Leuc mesenteroides* P-60 and *Lact. arabinosus* 17-5, were employed as the test organisms.

The inocula were prepared by transfer from the stock culture and grown for 17 hours at 37°C in a basal medium described below. The cells were centrifuged, washed and suspended in five volumes of saline. One drop of this suspension was used to inoculate each experimental tube.

Culture Medium and Assay Procedures—Compositions of the media employed for the experiments are shown in Table I.

Unless specifically stated, Medium I was used in all experiments. Composition of this medium was similar to that described by Henderson and Snell

(*7*), except for the modification as follows: vitamin B$_6$ compounds were supplied at the concentrations shown in Table I and L-isomers were used as amino acids except for alanine and serine. Medium II was approximately same as Medium I, but a number of amino acids were supplied as DL-form in this medium. Medium III was identical to that described by Steele and Sauberlich (*8*). Assays were carried out in 14×120 mm. tube and in a total volume of 2 ml.. Sterilization was accomplished by autoclaving the tubes for 3 minutes at 1 kilogram pressure. Because of the lability of pyridoxal when this vitamin is autoclaved with amino acids (*9*), vitamin B$_6$ compounds were autoclaved separately and then added to the sterilized basal medium. The tubes were incubated at 37°C and turbidimetric estimation of growth was made at suitable intervals directly in the culture tubes by use of the Hitachi model EPO-B electric photometer fitted with an adapter for this tube at 660 mμ; the results are expressed as optical density. The growth was also determined by titrating the acid produced with 0.1 *N* sodium hydroxide.

TABLE I

Composition of Basal Medium

Component	Medium I	Medium II	Medium III
	Amount per tube (per 1 ml. double strength medium)		
Glucose	40 mg.	40 mg.	50 mg.
Na-Acetate	2	2	40
Na-Citrate	40	40	—
NH_4Cl	6	6	6
KH_2PO_4	—	—	1.2
K_2HPO_4	10	10	1.2
$MgSO_4 \cdot 7H_2O$	1.6	1.6	0.4
$FeSO_4 \cdot 7H_2O$	0.08	0.08	0.02
$MnSO_4 \cdot H_2O$	0.32	0.32	0.04
NaCl	0.08	0.08	0.02
DL-Alanine	2000 μg.	2000 μg.	400 μg.
L-Arginine	400	400	480
L-Asparagine	—	—	800
L-Asparatic acid	1000	1000	200
L-Cysteine	—	—	100
L-Cystine	200	200	—
L-Glutamic acid	2000	2000	600
Glycine	200	200	200
L-Histidine·HCl	200	200	120
L-Leucine	200	400[a]	500[a]
L-Lysine·HCl	400	400	500
L-Methionine	200	400[a]	200[a]
L-Phenylalanine	200	400[a]	200[a]
L-Proline	200	200	200
DL-Serine	400	400	100
L-Threonine	200	400[a]	400[a]
L-Tryptophan	200	400[a]	80[a]
L-Tyrosine	200	200	200
L-Valine	200	400[a]	500[a]
Thiamine·HCl	2	1	1
Ca-Pantothenate	2	1	1
Riboflavin	2	1	1
Niacin	2	2	2
p-Aminobenzoic acid	0.4	0.2	0.2
Biotin	0.02	0.002	0.002
Foric acid	0,02	0.02	0.02
$Adenine \cdot H_2SO_4 \cdot 2H_2O$	20	20	20
$Guanine \cdot HCl \cdot 2H_2O$	20	20	20
Uracil	20	20	20
Xanthine	20	20	20
Pyridoxine·HCl	2	2	2
Pyridoxamine·2HCl	0.6	0.6	0.6
Pyridoxal·HCl	0.6	0.6	0.6

Unless specifically stated otherwise, Medium I was used in all experiments. The medium was adjusted to pH 6.0 before use.

[a] DL-Form was employed.

RESULTS

Growth Response of Str. faecalis R, Leuc. mesenteroides P-60 and Lact. arabinosus 17-5 to Isoleucine Isomers—The growth responses of the three micro-organisms to the isoleucine isomers in the presence or absence of vitamin B$_6$ compounds are summarized in Table II.

All three organisms responded to L-isoleucine both in the presence and absence of vitamin B$_6$ compounds, and even in the absence of this vitamin good growth occured not only with *Leuc. mesenteroides* P-60 and *Lact. arabinosus* 17-5 but also with *Str. faecalis* R which was known to require vitamin B$_6$. This growth is due to the presence of abundant DL-alanine in the basal meduin as previously noted (*10*).

It is of interest that among the three organisms studied, only *Lact. arabinosus* 17-5 was capable of utilizing all four isoleucine isomers in the presence of vitamin B$_6$ compounds, but not in the absence of this vitamin. This result is quite different from the results obtained by earlier investigators (*1*, *3*) that *Lact. arabinosus* 17-5 could not utilize D-isoleucine and D-*allo*isoleucine even in the medium containing vitamin B$_6$ compounds. On the other hand, *Str. faecalis* R and *Leuc. mesén-*

TABLE II

Growth Response of Lactic Acid Bacteria to Isoleucine Isomers

Isoleucine isomers	*Lact. arabinosus*		*Str. faecalis*		*Leuc. mesenteroides*	
	B_6 (+)	B_6 (−)	B_6 (+)	B_6 (−)	B_6 (+)	B_5 (−)
L-Isoleucine	+	+	+	+	+	+
L-*allo*Isoleucine	+	−	−	−	−	−
D-Isoleucine	+	−	−	−	−	−
D-*allo*Isoleucine	+	−	−	−	−	−

The concentration of isoleucine isomers was 2～100 μg. per 2 ml. tube. Incubation was carried out for 96 hours in Medium I of initial pH 6.8.

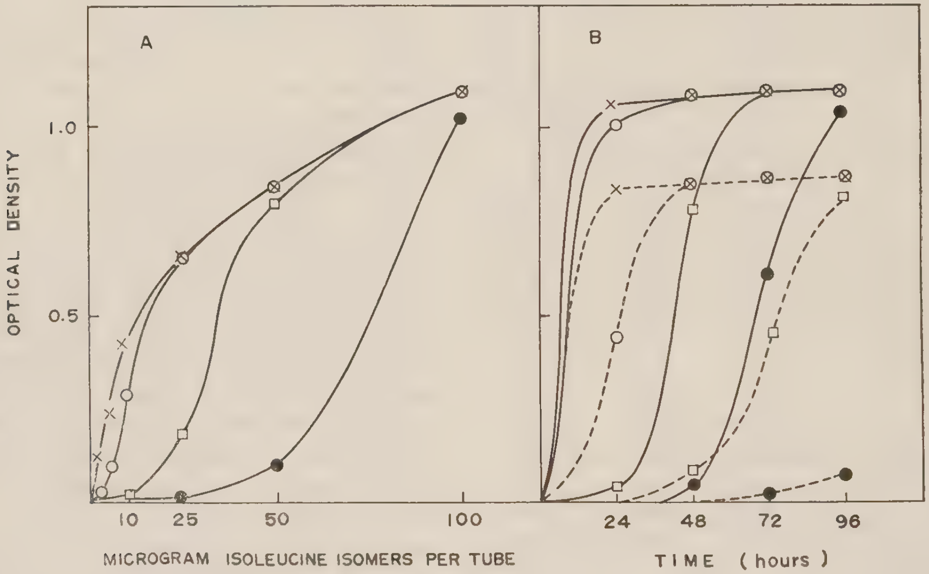

FIG. 1. Growth response of *Lact. arabinosus* to isoleucine isomers. Incubation was carried out in the medium of initial pH 6.0. A, incubated for 96 hrs.; B, contained 100 μg. (——) and 50 μg. (----) stereoisomers per tube.

×, L-isoleucine; ○, L-*allo*isoleucine; ●, D-isoleucine; □, D-*allo*isoleucine

teroides P-60 could not utilize L-*allo*isoleucine, D-isoleucine and D-*allo*isoleucine even in the medium containing vitamin B_6 compounds. On the other hand, *Str. faecalis* R and *Leuc. mesenteroides* P-60 could not utilize L-*allo*isoleucine, D-isoleucine and D-*allo*isoleucine even in the presence of vitamin B_6 compounds.

The response of *Lact. arabinosus* 17-5 to various concentrations of isoleucine isomers in the presence of vitamin B_6 compounds is shown in Fig. 1, A.

Approximately the same final level of growth was attained by L-*allo*isoleucine, D-isoleucine and D-*allo*isoleucine as that of L-isoleucine at higher concentrations. However, it was observed that maximum growth with L-isoleucine appeared after incubation for 24 to 48 hours, and the maximum growth with L-*allo*isoleucine and D-*allo*isoleucine was usually attained respectively in 48 to 72 hours and 72 to 96 hours, in other words, about 24 hours and 48 hours longer than the time

time required for L-isoleucine. Response to D-isoleucine was lowest among the stereoisomers tested, and maximum growth was reached 72 hours later than with L-isoleucine. (Fig. 1, B.).

Effect of pH of the Medium on the Response of Lact. arabinosus 17-5 to Isoleucine Isomers—The growth response with four isomers of isoleucine was compared at pH 6.8, 6.0, 5.3 and 4.8.

Although the growth with L-isoleucine was scarcely affected by initial pH of the medium, the adjustment of the initial pH of the medium to 6.0 or 5.3 resulted in better growth acceleration with stereoisomers than that of pH 6.8. Growth response in the medium adjusted to pH 6.8 was lower than that of pH 4.8. As shown in Fig. 2, growth-time curves obtained at pH 6 and pH 6.8 were compared at the concentration of 100 μg. of the isomers per 2 ml. tube.

By lowering the initial pH of the medium, the lag in the growth response to D-isoleucine and D-*allo*isoleucine was markedly diminished. When the medium was adjusted to pH 6.8, appreciable growth was not observed at even high concentrations of D-isoleucine.

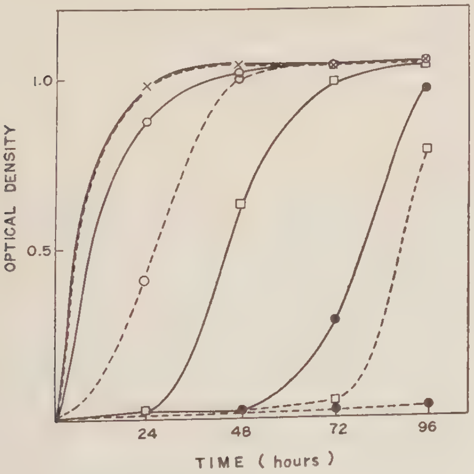

Fig. 2. Growth response of *Lact. arabinosus* to isoleucine isomers on the medium of pH 6.0 (solid curve) and pH 6.8 (broken curve). A concentration of the isomers was 100 μg. per tube. ×, L-isoleucine; ○, L-*allo*isoleucine; ●, D-isoleucine; □, D-*allo*isoleucine

Effect of Composition of the Medium on the Response of Lact. arabinosus 17-5 to Isoleucine Isomers—The utilization of isoleucine isomers by *Lact. arabinosus* 17-5 is dependent on the composition of the basal medium. With each stereoisomers of isoleucine, the growth response of *Lact. arabinosus* 17-5 in Medium I, Medium II and Medium III was compared. The former is the medium primarily employed by the author and the latter two media are considered to be that employed by Meister (*3*), who reported that D-isoleucine and D-*allo*-isoleucine are inactive for the growth of *Lact. arabinosus* 17-5. As shown Fig. 3, employment of Medium II and Medium III containing mainly DL-form of amino acids resulted in slower growth response with D-isoleucine and D-*allo*isoleucine than that of Medium I which contained primarily L-form of amino acids.

At the concentration of 25 μg. of the isomers per 2 ml. tube, D-*allo*isoleucine supported the growth only in Medium I but not in the other media, and D-isoleucine showed no activity for the growth in all three media under these conditions. On the other hand, at the concentration of 100 μg. per 2 ml. tube, the organism became to utilize not only D-*allo*isoleucine but also D-isoleucine in Medium II after the long incubation such as 144 hours, however its growth was markedly slower than that in Medium I.

The difference of the conditions for cultures, namely, the concentrations of the isomers tested, incubation period and the composion of the media seems to account for the discrepancy between the results obtained by the author and the earlier reports that D-isoleucine and D-*allo*isoleucine did not support the growth of *Lact. arabinosus* 17-5. From the data obtained in this experiment, it is considered that if an incubation period longer than 144 hours and a concentration higher than 50 μg. per 2 ml. tube were employed, D-isoleucine and D-*allo*isoleucine should be also utilized by *Lact. arabinosus* 17-5 even under the conditions employed by Meister, who used approximately the same medium as Medium II.

Effect of Leucine and Valine on the Response of Lact. arabinosus 17-5 to Isoleucine—Concern-

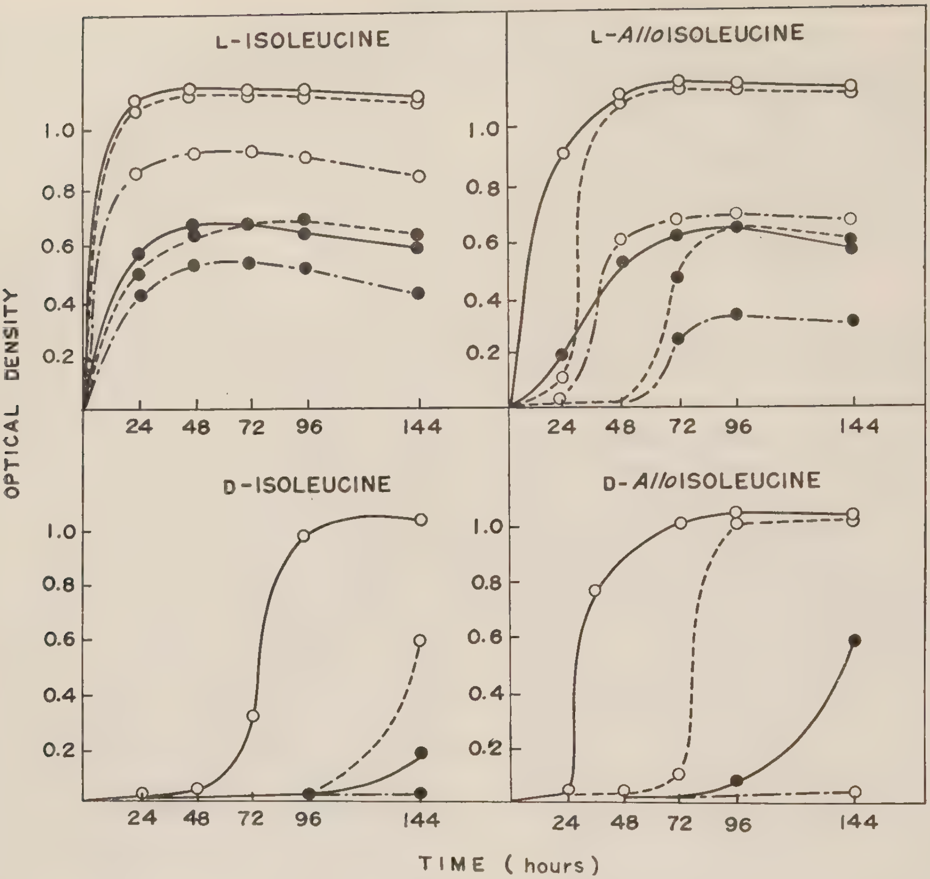

FIG. 3. Effect of the varied basal media on the growth response of *Lact. arabinoses* to isoleucine isomers.
Isoleucine isomers of 100 μg. (○) and 25 μg. (●) per tube were incubated in Medium I (——), Medium II (---) and Medium III (-·-·-) at 37°C.

ing the influence of the components of the media upon the utilization of isoleucine isomers, it was considered that the growth response is affected by the concentrations of leucine and valine present in the medium. As shown in Table III, excessive amounts of leucine and valine, especially the former, inhibited the utilization of isoleucine isomers. For example, higher concentrations of L- or DL- leucine than 400 μg. per 2 ml. tube completely suppressed the response to D-isoleucine and D-*allo*isoleucine. It appears that this inhibition is affected by the concentration of leucine and not by the difference of optical configuration such as L- or DL- form of leucine. When 400 μg. of DL-leucine and DL-valine per 2 ml. tube was used in Medium I, the utilization of isoleucine isomers was very poor as shown in Table III. Therefore, difference of the utilization of isoleucine isomers in Medium I and in Medium II is considered chiefly due to the amounts of leucine and valine in the media employed. Decreasing the concentrations of L-leucine and L-valine in the basal medium form the standard level of 200 μg. per 2 ml. tube to 100 μg. accelerated the growth with isoleucine isomers, especially D-isoleucine and D-*allo*-isoleucine.

Influence of Pyridoxine, Pyridoxal and Pyrido-

TABLE III

Effect of Valine and Leucine on the Response of Lact. arabinosus to Isoleucine Isomers

Time	Isoleucine isomers	Add. Leu. Val. μg./tube	Growth response at various concentrations of leucine and valine in medium I												
			L-Val 200						L-Leu 200						DL-Val a) 400
			L-Leu			DL-Leu			L-Val			DL-Val			DL-Leu
		μg./tube	100	200	1000	200	400	1000	100	200	1000	200	400	1000	400
50 hours	L-Ileu	25	730	710	690	730	720	705	720	710	700	720	720	710	710
		50	860	860	860	865	860	855	860	865	855	860	860	855	860
		100	1050	1090	1070	1090	1090	1085	1080	1090	1080	1090	1070	1090	1090
	L-*allo* Ileu	25	660	640	175	655	625	390	640	645	570	650	645	570	620
		50	860	850	760	850	850	820	850	840	840	845	840	840	850
		100	1050	1050	1060	1060	1050	1060	1020	1060	1020	1040	1060	1020	1040
	D-Ileu	25	20	0	0	0	0	0	0	0	0	0	0	0	0
		50	310	18	0	10	0	0	15	13	0	0	0	0	10
		100	1050	200	0	580	30	0	420	200	50	200	180	50	25
	D-*allo* Ileu	25	180	25	0	30	0	0	35	38	0	30	20	0	10
		50	870	135	0	150	30	0	185	135	50	180	120	50	30
		100	1020	1030	0	1030	200	10	1040	5025	920	1040	1005	940	315

The recorded values are optical density (×1000)

a) This is the same concentrations as that of Medium II.

xamine on the Utilization of Isoleucine Isomers by Lact. arabinosus 17-5—From the data shown in Table II, it appears that *Lact. arabinosus* 17-5 can not utilize isoleucine isomers when vitamin B_6 compounds are absent in the basal medium. Experiments were then designed to determine which is the most active among pyridoxine, pyridoxal and pyridoxamine for this utilization. Details of the results comparing the activities of these compounds for the growth response with isoleucine isomers are given in Table IV.

Pyridoxamine was most effective for the utilization of the isomers and pyridoxal followed. Activity of pyridoxine was the lowest and it is effective only for the utilization of L-*allo*isoleucine under the conditions employed by the author. The addition of a little amount of pyridoxamine alone permits the same good growth as that in Medium I. On the other hand, it seems that presence of excessive amounts of pyridoxine or pyridoxal inhibited the utilization of isoleucine isomers.

Comparison of the Utilization of Isoleucine Isomers by Ordinary Strain of Lact. arabinosus 17-5 and the Strain Adapted to Isoleucine Isomers—As mentioned above, *Lact. arabinosus* 17-5 is capable to grow in Medium I containing either one of the stereoisomers of isoleucine in place of L-isoleucine, when vitamin B_6, especially pyridoxamine, is present. Therefore, this strain was successively cultured several times in the agar medium consisting of the same composition as Medium I supplemented with 2% agar, and the strain was adapted to respective isoleucine isomers. Inoculum of this adapted strain was cultured in the same medium omitting agar for 17 to 20 hours. From the experiments employing this inoculum, it was found that the strain cultured successively in the medium containing one of the other isoleucine isomers in

TABLE IV

The Effect of Vitamin B_6 on the Utilization of Isoleucine Isomers by Lact. arabinosus

Concentration of vitamin B_6 µg./tube		Growth with isoleucine isomers			
		L-Isoleucine	L-*allo*Isoleucine	D-Isoleucine	D-*allo*Isoleucine
None		575	0	0	0
Pyridoxine	10	660	0	0	0
	100	870	20	0	0
	1,000	1030	600	0	0
	10,000	1030	62	0	0
Pyridoxal	0.01	865	67	0	5
	0.05	1000	395	10	32
	0.1	1040	785	26	48
	1	1050	1020	153	930
	10	1050	1040	840	1040
	100	1050	1040	7	1010
Pyridoxamine	0.001	725	91	0	10
	0.01	1025	265	17	25
	0.05	1050	980	115	342
	0.1	1050	1030	350	710
	0.5	1060	1055	850	1010
	1	1060	1050	1040	1040

Isoleucine isomers of the concentrations 100 µg./2 ml. tube were incubated for 72 hours at 37°C. The recorded values are optical density (×1000).

place of L-isoleucine was able to utilize isoleucine isomers more rapidly than ordinary strain cultured in the medium containing L-isoleucine.

Although the growth responses to L-isoleucine with ordinary strain and with the adapted strain were nearly equal, the growth responses to the other isomers, especially to D-isoleucine and D-*allo*isoleucine, were better with adapted strain than with ordinary strain. The adaptation of *Lact. arabinosus* 17-5 to D-isoleucine or D-*allo*isoleucine was most effective on the utilization of the isomers of isoleucine and the adaptation to L-*allo*isoleucine was not so effective.

A comparison of the growth-time curves of ordinary strain and of the strain adapted to D-isoleucine is shown in Fig. 4.

Although the appreciable growth of ordinary strain with D-isoleucine and D-*allo*-

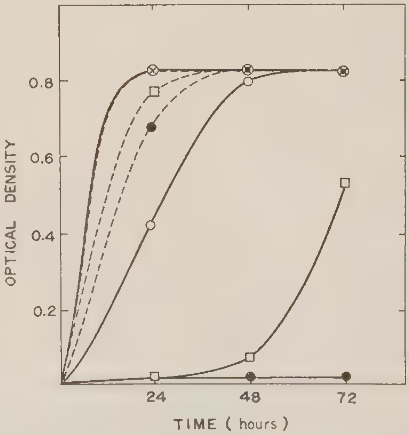

FIG. 4. Growth responses of *Lact. arabinosus* adapted for D-isoleucine medium and of ordinary strain to 50 µg. isoleucine isomers.

×, L-isoleucine; ○, L-*allo*isoleucine; ●, D-isoleucine; □, D-*allo*isoleucine. ——, ordinary strain; ----, adapted strain.

isoleucine was not observed during this incubation period, the adapted strain reached to the maximum grouth with respective isomers in 24 to 48 hours. It was also found that the strain adapted to D-isoleucine showed marked acceleration on the responce not only to D-isoleucine but also to L-*allo*isoleucine and D-*allo*isoleucine. The adapted strains to L-*allo*isoleucine and D-*allo*isoleucine also indicated no specificity. Namely, the strains adapted to L-*allo*isoleucine or D-*allo*isoleucine also utilized rapidly all the other stereoisomers. This characteristic of the adapted strains was not lost completely after the organism was cultured once in the ordinary medium containing L-isoleucine.

DISCUSSION

From the experiments on the growth response of *Str. faecalis* R and *Leuc. mesenteroides* P-60 to isoleucine isomers, it appears that only L-isoleucine among the four stereoisomers can be assayed by employing *Str. faecalis* R *and Leuc. mesenteroides* P-60, since these organisms do not utilize the other stereoisomers both in the presence and absence of vitamin B_6 compounds in the medium. When *Lact. arabinosus* 17-5 is employed as the test organism, L-isoleucine can be assayed in the absence of vitamin B_6, but not in the presence of vitamin B_6, because this organism utilizes L-*allo*isoleucine, D-isoleucine and D-*allo*isoleucine in the medium containing this vitamin. Therefore, the determination of L-isoleucine in the mixtures containing other isoleucine isomers such as synthetic preparations and commercial samples, must be carried out with attention on this point. If *Lact. arabinosus* 17-5 shows the same response to L-*allo*isoleucine as to L-isoleucine and this organism dose not utilize D-isoleucine and D-*allo*isoleucine as reported by Meister (*3*), it is expected that only L-*allo*isoleucine in the mixture of isoleucine isomers may be determined by the difference between the assays in the medium containing vitamin B_6 and that in the absence of this vitamin. This is, however, impossible, because *Lact. arabinosus* 17-5 is capable to utilize D-isoleucine and D-*allo*isoleucine besides L-*allo*isoleucine as revealed by present experiments. Although, the findings obtained by the present experiments suggest that determination of the total amounts of synthetic isoleucine and *allo*isoleucine mixtures becomes possible by the method of microbioassay. The advantageous microbiological method of the determination of total isoleucine isomers by employing the adapted strain of *Lact. arabinosus* 17-5 obtained in this experiment will be described later in the following reports.

The present studies revealed that discrepancy between the results obtained by the author and previous reports concerning the utilization of D-isoleucine and D-*allo*isoleucine by *Lact. arabinosus* 17-5, is due to the difference of conditions of cultures.

It is considered that the inhibition of the utilization of isoleucine isomers by leucine and valine is competitive; because, in all cases, increasing the concentration of isoleucine isomers reversed the inhibition.

From comparative studies on the effect of vitamin B_6 compounds for the utilization of isoleucine isomers by *Lact. arabinosus* 17-5, it is confirmed that pyridoxamine is the active form of vitamin B_6 for *Lact. arabinosus* 17-5. This conclusion is also drown from the experiment, in which the activities of vitamin B_6 compounds in replacing alanine for this organism are investigated (*10*). Probably, the slight activity of pyridoxine for the utilization of L-*allo*isoleucine is due to conversion of a minute amount of pyridoxine to pyridoxamine or pyridoxal by the reaction with other constituents of the medium during the incubation or by enzymatic conversion during the growth of the organism, although vitamin B_6 compounds were autoclaved separately to avoid destruction and conversion of this vitamin. It seems that the minute quantities of pyridoxamine or pyridoxal formed from pridoxine are not sufficient to provide the amount required for the utilization of D-isoleucine and D-*allo*isoleucine, but are sufficient for the utilization of L-*allo*isoleucine.

SUMMARY

Microbiological studies on the utilization of isoleucine isomers by lactic acid bacteria were carried out and the results obtained are summarized as follows:

1. *Str. faecalis* R and *Leuc. mesenteroides* P-60 are unable to utilize L-*allo*isoleucine, D-isoleucine and D-*allo*isoleucine both in the presence or absence of vitamin B_6 compounds.

2. Among the three organisms studied, only *Lact. arabinosus* 17-5 is capable of utilizing all four isoleucine isomers in the presence of vitamin B_6 compounds, but not in the absence of this vitamin.

3. By lowering the initial pH of the medium, the growth of *Lact. arabinosus* 17-5 to isoleucine isomers is accelerated.

4. Excessive amounts of leucine inhibit the utilization of isoleucine isomers by *Lact. arabinosus* 17-5.

5. It is considered taat discrepancy between the results obtained by the author and previous reports concerning the utilization of D-isoleucine and D-*allo*isoleucine is due to the difference of conditions of cultures.

6. Pyridoxamine is most effective for the utilization of the stereoisomers of isoleucine by *Lact. arabinosus* 17-5.

7. The strain cultured successively in the medium containing one of the other isomers in place of L-isoleucine is able to utilize isoleucine isomers more rapidly.

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Microbiological and Enzymatic Studies on the Isoleucine Isomers

II. Microbiological Determination of L-Isoleucine and Total Isoleucine Isomers

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(Received for publication, May 1, 1961)

Existence of *allo*-form in the amino acids possessing two asymmetric carbons gives rise to formidable problems for the synthesis and optical resolution of the amino acid. There has been, however, no simple and convenient method for determination of the proportion contaminated with *allo*-form in the amino acid such as isoleucine.

Consequently, establishment of an advanced microbiological method for the assay of *allo*-form in synthetic isoleucine mixtures, was a purpose of this series. This purpose was attained by an application of the results of the fundamental studies presented in the previous report (*1*) that *Lactobacillus arabinosus* 17–5 cultured successively in the medium containing D-isoleucine in place of L-isoleucine is able to utilize all stereoisomers of isoleucine more rapidly in the presence of vitamin B_6 compound.

Studies on the bioassay of L-isoleucine with lactic acid bacteria have been reported by a number of authors (*2–9*), but microbiological method of the determination of the total amount of isoleucine isomers has not yet been reported. These reports showed that *Lact. arabinosus* 17–5 can be employed for the determination of L-isoleucine in protein hydrolysate (*3*, *4*, *6*) but not for the determination of synthetic isoleucine preparations, because the stereoisomers are partially utilized by this organism (*10–13*). Although, these previous studies were carried out under somewhat ambiguous conditions and not suitable for the quantitative interpretation of the results.

In contrast with these earlier reports, it was found from the present experiment that *Lact. arabinosus* 17–5 is very superior strain as the test organism, because contents of both L-isoleucine and total isoleucine isomers can be determined by this organism under appropriate conditions. Namely, it becomes possible to determine total isoleucine isomers in the mixture of a number of amino acids by using the basal medium containing vitamin B_6 compounds and the determination of L-isoleucine alone in the mixtures can be also made by the same assay procedure in the medium without vitamin B_6 compounds.

EXPERIMENTALS

Stereoisomers of isoleucine and assay procedure employed in this experiment were similar to those given in the preceding paper (*1*).

Basal Medium—The composition of the basal medium used in this experiment was the same as that of Medium I described in previous report (*1*), but slightly modified as follows: amounts of valine and leucine were decreased to 10 mg. per 100 ml. double strength medium. Because, the presence of valine and leucine inhibited the utilization of isoleucine isomers by *Lact. arabinosus* 17–5 as revealed by the previous experiment (*1*). Since the adjustment of the initial pH of the medium to 6.0 resulted in more rapid growth with the stereoisomers of isoleucine than that of pH 6.8 or 7.0, as described in the previous part by one of the authors, the medium was adjusted to pH 6.0 before use. When the excessive amounts of valine and leucine are contained in the samples, omission of valine and leucine from the basal medium is required for the determination. For the purpose of determination of the total amounts of isoleucine isomers, vitamin B_6 compounds are added to the basal medium and this vitamin is omitted for the determination of L-isoleucine. Of the vitamin B_6 compounds, pyridoxamine is most effective for the growth of *Lact. arabinosus* with all isoleucine isomers.

Organism—Lact. arabinosus 17–5 cultured in the ordinary agar medium containing peptons, meat extract and yeast extract was transfered to the basal medium supplemented with 200 µg. of D-isoleucine per 2 ml. This culture was incubated for about 3 days at 37°C until good growth occurred, and then the culture was transferred to stab culture in the agar medium of the same composition. The stab cultures were successively transferred about ten times to adapt the microorganism to D-isoleucine. This adapted strain was used as the test organism. Stock cultures of the adapted strain to D-isoleucine transferred biweekly, and were stored in a refrigerator until the next transfer.

Inoculum—Basal medium supplemented with 200µg. of D-isoleucine per 2 ml. was used for the growth of inoculum. After about 20 hours incubation at 37°C the cells were centrifuged, resuspended in 5 ml. of 0.9% sodium choride solution, recentrifuged, and again suspended in 10 ml. of sterile saline. Each tube was inoculated with one drop of this cell suspension.

RESULTS

Assay of Total Isoleucine Isomers—A comparison of the response curves of L-isoleucine and D-isoleucine in the presence of vitamin B_6 compounds is given in Fig. 1.

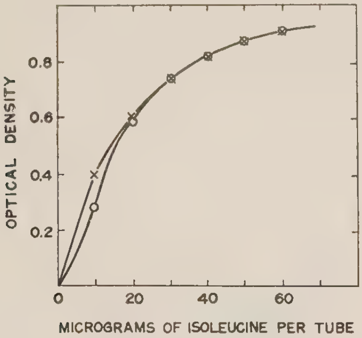

FIG. 1. Typical standard curves for L-isoleucine (×) and D-isoleucine (○) in the basal medium containing vitamin B_6. Time of incubation was 48 hours.

The same response was obtained with the both isomers of isoleucine at levels higher than 25 µg. per tube. At lower concentrations, the growth response with L-isoleucine was the highest among the four stereoisomers of isoleucine, while L-*allo*isoleucine and D-*allo*isoleucine followed, and D-isoleucine was the lowest.

The growth responses with the samples containing DL-isoleucine and DL-*allo*isoleucine in varied proportions were compared with that of L-isoleucine. These results are shown in Table I.

TABLE I

Growth Response of Adapted Lact. arabinosus 17–5 for Various Samples

Time hrs.	Sample No.	Optical Density (×100)					
	Sample No.	L	0	3	5	8	10
	DL-ILeu	(L-ILeu)	10	7	5	2	0
	DL-*allo* ILeu		0	3	5	8	10
	µg./tube						
22.5	10	335	260	260	255	255	238
	20	540	505	505	500	495	490
	30	675	670	670	670	665	655
	40	740	740	740	740	740	735
	60	860	860	855	860	855	855
48.5	10	400	370	370	365	370	370
	20	630	610	610	610	595	600
	30	760	760	760	760	755	760
	40	800	805	805	805	805	805
	60	910	910	910	915	910	910
71.0	10	430	415	415	410	415	415
	20	675	655	660	655	635	635
	30	760	760	760	760	755	755
	40	790	790	800	800	790	800
	60	900	900	900	900	900	900

At levels lower than 20 µg. per tube the response with the mixtures of DL-isoleucine and DL-*allo*isoleucine were lower than with L-isoleucine, although at higher concentrations the same response was obtained with the mixtures as that with L-isoleucine after 48 hours incubation. The results obtained in these experiments indicate that the total amounts of isoleucine isomers in the mixture of synthetic preparations can be determined at concentrations of 25 µg. to 60 µg. per tube using the standard curve of L-isoleucine. Similar results were also obtained with the method by titrating the acid produced with 0.1 *N* sodium hydroxide.

Assay of L-*Isoleucine*—It has been previously found by one of the authors that ordinary strain of *Lact. arabinosus* 17–5 can utilize all four stereoisomers of isoleucine in the presence of vitamin B_6 compounds, but utilizes only L-isoleucine in the absence of vitamin B_6. The same fact as with ordinary strain was also ascertained with the adapted strain which was used for the determination of total amounts of isoleucine isomers in present experiments. Namely, as indicated in Table II, the adapted strain utilized only L-isoleucine, but not the other isomers in the absence of this vitamin.

Therefore, when vitamin B_6 compounds are omitted from the composition of the basal

TABLE II

Growth Response to Isoleucine Isomers in the Presence or Absence of Vitamine B_6 Compounds

Medium	Optical Density (×1000)			
	L-Isoleucine	L-*allo*Isoleucine	D-Isoleucine	D-*allo*Isoleucine
Complete	1080	1080	1075	1085
Vitamin B_6 omitted	940	10	20	10

The concentration of isoleucine isomers was 100 μg. per tube. Incubation period was 48 hours.

TABLE III

L-*Isoleucine Content of* DL-*Isoleucine*—DL-*alloIsoleucin Mixtures*

Sample			Test Organism					
			Adapted Strain2)		*Str. faecalis* R3)		*Leuc. mesenteroides* P-603)	
Sample1) No.	Added μg. per tube	Calculated (L-ILeu) μg. per tube	Found μg. per tube	Recovery per cent	Found μg. per tube	Recovery per cent	Found μg. per tube	Recovery per cent
0	10	5.0	4.9	98	4.9	98	5.1	102
	30	15.0	15.6	104	15.0	100	14.6	97
	50	25.0	24.0	96	24.0	96	24.8	99
3	10	3.5	3.5	100	3.5	100	3.6	103
	30	10.5	10.8	103	10.5	100	10.5	100
	50	17.5	17.0	97	17.1	98	17.0	97
5	15	3.8	3.8	100	3.7	97	3.9	103
	30	7.5	7.9	105	7.3	97	7.5	100
	60	15.0	14.8	99	15.0	100	14.8	99
8	30	3.0	3.1	103	3.0	100	3.1	103
	60	6.0	6.1	102	6.1	102	6.1	102
10	60	0	0	—	0.2	—	0.1	—

1) The proportion of DL-isoleucine to DL-*allo*isoleucine was shown in Table I.

2) Assy was carried out in the absence of vitamin B_6.

3) Assy was carried out in the presence of vitamin B_6 by ordinary method. Time of incubation was 24 hours.

medium, only L-isoleucine can be determined among four isoleucine isomers with this adapted strain by the essentially same procedure as that of determination of total isoleucine.

As shown in Fig. 2, satisfactory standard curve was obtained for L-isoleucine in a vitamin B_6 free medium when the adapted strain was used as the test organism.

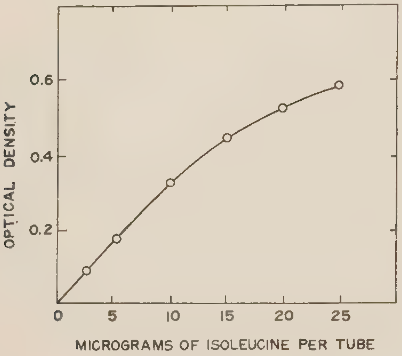

FIG. 2. Typical standard curve for L-isoleucine of vitamin B_6. Time of incubation was 24 hours.

L-Isoleucine contents of the samples of DL-isoleucine–DL-*allo*isoleucine mixtures used in the above experiment (Table I) were determined with this standard curve. The results are shown in Table III.

These values obtained with the adapted strain was in good agreement with the values obtained by ordinary bioassay employing *Str. faecalis* R and *Leuc. mesenteroides* P-60.

Application of the Assay to Amino Acid Mixtures—The total contents of isoleucine isomers and the contents of L-isoleucine in the mixture prepared by blending several amino acids were determined. In each case, the recoveries were within 95–105 per cent. The data obtained in this expreiment indicate that this assay method can be preformed even in the presence of many other amino acids.

DISCUSSION

It has been well known that there are three available forms in commercial synthetic isoleucine preparations, *i. e.* (a) DL-isoleucine, (b) DL-isoleucine–DL-*allo*isoleucine, and (c) L-isoleucine–D-*allo*isoleucine. Although L-isoleucine can be assayed by ordinary bioassay procedure, troublesome procedure such as chemical assay after chromatographic separation has been required for the determination of total isoleucine isomers, especially in the presence of other amino acids.

To solve this problem, application of the facts previously revealed by the author that the strain of *Lact. arabinosus* 17–5 adapted to D-isoleucine could readily utilize all stereoisomers of isoleucine, was studied in detail, and novel microbiological assay procedure for the determination of total isoleucine isomers has been accomplished.

The adapted strain of *Lact. arabinosus* 17–5 which was cultured successively about fifteen times to twenty times in the basal agar medium supplemented with D-isoleucine was used as test organism in this experiment. However, the strain transferred successively more than ten times can be used for the assay.

The determination of L-isoleucine also in the mixture of isoleucine isomers can be made by the essentially same procedure as that of determination of total isoleucine in the medium without vitamin B_6 compounds. Therefore, from the difference of the values assayed in the medium containing vitamin B_6 and in the medium omitted this vitamin, it is possible to determine *allo*-form in the mixture of synthetic isoleucine preparation. The fact that this assay method can be performed even in the presence of many other amino acids is of great advantage to practical application.

SUMMARY

The microbiological determination of L-isoleucine and the total amount of isoleucine isomers by empoying the adapted strain of *Lact. arabinosus* 17–5 is described.

This assay method is found to be very convenient and satisfactory for the determination of total isoleucine isomers in amino acids mixtures and this method also can be applied to the assay of the proportion contaminated with *allo*-form in synthetic isoleucine preparations.

The authors wish to thank Prof. S. Akabori and Assist. Prof. Y. Izumi, Osaka University, for their interest and helpful advice throughout this work. The authors are also greatly indebted to Mr. T. Hirabayashi, Representative Director of the Tanabe Seiyaku Co. Ltd., Dr. M. Fujisawa, Research Executive and Dr. N. Sugimoto. Director of Osaka Research Laboratory, for continued encouragement and assistance.

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Studies of Hβ-Proteinase of Habu Snake Venom, with Special Reference to Substrate Specificity and Inhibitory Effect of Serum

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(Received for publication, May 12, 1961)

In previous papers (*1*, *2*), it was reported that there exist two proteolytic enzymes in Habu snake venom, that is, Hα- and Hβ-proteinase. In addition to these enzymes another proteinase has been recently found which shows the optimal pH at 8.4 (*3*). Hα-proteinase was partially purified by ammonium sulfate fractionation and calcium phosphate gel adsorption, and Hβ-proteinase was also obtained in an ultracentrifugally homogeneous state by a column chromatographic technique using Amberite IRC-50 (*4*).

An intramuscular injection of the purified Habu proteinases into experimental animals caused necrosis of muscle with hemorrhage, which is the most serious symptom resulting from Habu bite, though here the extent of the necrosis was less than that in the case of the crude venom (*5*, *10*). The proteinase obtained from other sources such as trypsin and S-proteinase caused also myolysis and hemorrhage at the site of the injection. However, the reaction was far weaker than that in the case of Habu proteinases. The difference in the extent of the pathological changes caused by various proteolytic enzymes may be attributed to substrate specificity, low activity on natural muscle protein, stability of the enzymes and inhibitory effects of serum. The present paper deals with the substrate specificity of Hβ-proteinase and an inhibitory effect of serum on various proteinases for the better understanding of the mechanisms whereby the necrosis of muscle with hemorrhage is caused.

MATERIALS AND METHODS

Anti-*Habu* venom from horse was kindly supplied by Dr. Y. Sawai of the Institute for Infectious Diseases, University of Tokyo. Crystalline trypsin was a product of Mochida Pharmaceutical Manufacturing Co., LTD. Crystalline proteinase from *Streptomyces griseus* was generously donated by Dr. M. Nomoto of the Institute of Physical and Chemical Research, Tokyo. Whale insulin was obtained from Shimizu Pharmaceutical Co., LTD. Glycyl and phenylalanyl chain of the insulin were prepared by performic acid oxidation according to the method of Sanger (*6*, *7*). Hβ-proteinase used was the preparation purified from Habu (*Trimeresurus flavoviridis*) snake venom according to the method described in the previous paper (*4*).

The measurement of the proteolytic activity when casein was used as substrate, was described in the previous report (*4*). Hydrolysis of glycyl and phenylalanyl chain by Hβ-proteinase was determined by the alcohol-alkali titration method. The reaction mixtures with 0.25 ml. of the total volume contained Hβ-proteinase, substrate and 8 μmole Na_2HPO_4, the pH of which was adjusted to 8.1. The reaction was carried out at 35°C, stopped by addition of 1 ml. of absolute ethanol, titrated potentiometrically with constant shaking with 0.02 *N* NaOH containing 80% ethanol and then concentrated to about 0.2 ml. *in vacuo*. The resulting peptides were then treated with DFB according to the ordinary method (*8*, *9*), hydrolyzed with 6 *N* HCl in a sealed tube at 105°C and the DNP-amino acids were fractionated with ether and then DNP was removed by sublimation. The DNP-amino acids were then authentically identified by two dimensional paper chromatography with use of *n*-butanol saturated with 0.1% ammonia and 1.5 *M* phosphate as the two solvents. For the quantitative determination of the N-terminal residues, the yellow spots on the paper were cut off and eluted separately

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This research was supported in part by a research grant of the Institute for Protein Research, Osaka University. The abreviations used are; DNP-, dinitrophenyl; FDB, fluoro-2,4-dinitrobenzene; S-proteinase, proteinase from *Streptomyces griseus*.

with warm water and the optical density at 360 mμ was determined for each extract.

Hydrolysis of di- and tripeptides as substrate was measured according to the alcohol-alkali titration method and/or one dimensional paper chromatographic technique.

RESULTS

Effect of Serum on Some Proteinases—Inhibitory effects of normal horse and rabbit sera on Hβ-proteinase, S-proteinase and trypsin were investigated. It is obvious from Fig. 1a and 1b that both normal sera of horse and rabbit scarcely inhibit Habu proteinase at all, whereas they are quite inhibitory to S-proteinase and especially to trypsin. A similar result was obtained with human serum.

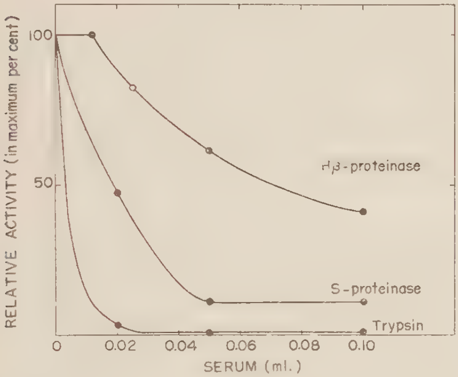

FIG. 1a. Effect of normal horse serum. The incubation mixtures with 2.0 ml. of total volume contained 50 μmoles of veronal buffer of pH 9.0, 20 mg. of casein and 100 μg. of Hβ-proteinase or 20 μg. of S-proteinase. For the assay of trypsin, 125 μmoles of phosphate buffer of pH 7.5 and 25 μg. of the enzyme were used. The reactions were carried out at 37°C for 10 minutes.

The anti-Habu serum showed a far greater inhibitory effect on Hβ-proteinase than the normal horse serum, as well as on Hα-proteinase already reported in the previous paper (*1*) (Fig. 2).

Fig. 3 shows the complete inhibition of Hβ-proteinase by the addition of the anti-Habu serum to the incubation mixture in the course of the reaction. This fact, therefore, suggested that an application of the antiserum which contains specific antibody for Hβ-proteinase, is very useful for therapeutic treatment of the local lesions due to Habu proteinases.

Proteolytic Activity of Hβ-proteinase and S-proteinase on Casein—The extent of the digestion of casein as substrate by Hβ-proteinase and S-proteinase was investigated, as seen in Fig. 4.

For curve I, the reaction was initiated by the addition of 30 μg. of Hβ-proteinase

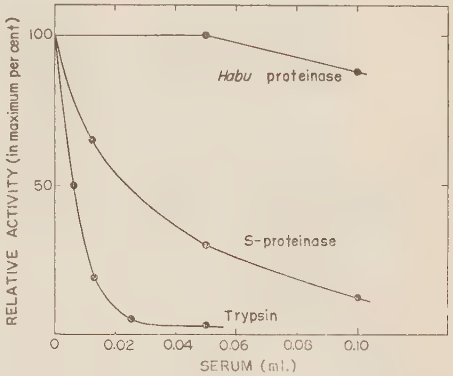

FIG. 1b. Effect of normal rabbit serum. Assay conditions were almost the same as in Fig. 1a except the use of 300 μg. of crude venom for the determination of the proteolytic activity of Habu snake venom.

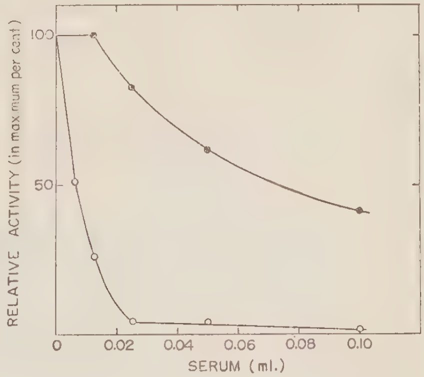

FIG. 2. Effect of serum on Hβ-proteinase. The reaction mixtures contained 0.5 ml. of 0.1 *M* veronal buffer of pH 9.0, 0.1 mg. of enzyme, 0.2 ml. of diluted serum and 1 ml. of 2% casein. The total volume was 2.0 ml. The reaction was carried out at 37°C for 20 min.

—●— normal horse serum

—○— anti-Habu venom serum from horse

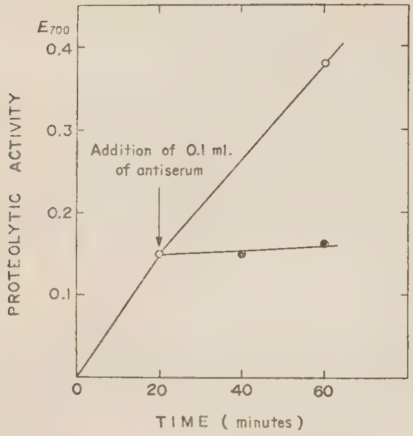

Fig. 3. Effect of antiserum on reaction rate. The incubation mixture with the total volume of 2.0 ml. contained 0.5 ml. of 0.1 *M* veronal buffer of pH 9.0, 100 μg. enzyme and 20 mg. casein. Reaction temp., 37°C

—○— without antiserum

—●— with 0.1 ml. of antiserum

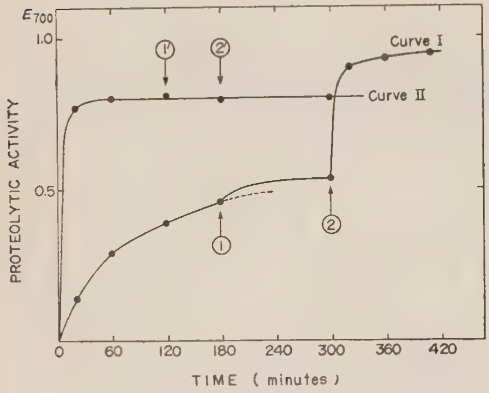

Fig. 4. Digestion of casein by Hβ- and S-proteinase. In every test tube with 1.0 ml. of the total volume were contained 5 mg. of casein, 30 μ moles of veronal buffer of pH 9.0 and enzymes and incubated at 37°C. After deproteinization with TCA, peptides and amino acids in the supernatant were determined colorimetrically as described in the text.

For curve I, the reaction was initiated on addition of 30 μg. of Hβ-proteinase. Addition at Arrow 1, 15 μg. of Hβ-proteinase, Arrow 2, 50 μg. of S-proteinase. For curve II, the reaction was initiated on addition of 50 μg. of S-proteinase. Addition at Arrow 1′, 25 μg. of S-proteinase, Arrow 2′, 50 μg. of Hβ-proteinase.

and 50 μg. S-proteinase was added to the incubation mixture when the addition of another 15 μg. of Hβ-proteinase in the course of reaction showed no further increase in activity. A rapid increase in proteolysis of the casein which had been completely digested by Hβ-proteinase was observed. On the other hand, when the reaction was initiated by the addition of 50 μg. of S-proteinase, the extent of the digestion reached its maximum in one hour and thereafter remained constant. Even on adding another 25 μg. of S-proteinase and then 50 μg. of Hβ-proteinase, the proteolysis of casein did not increase. Therefore, it is concluded from these results that the activity of S-proteinase in solubilyzing casein into trichloroacetic acid is far greater than that of Hβ-proteinase.

Proteolytic Activities of Hβ-proteinase on Some Proteins—Table I shows the activity of the proteinase on native and heat-treated hemoglobin and serum albumin compared with that on heat-treated casein.

Table I

Proteolytic Activities of Hβ-proteinase on Some Proteins

Substrate	Relative Activity	
	untreated	heat-treated
casein		100
hemoglobin	21	40
serum albumin	8	10

The incubation mixture with 2.0 ml. of the total volume contained 0.5 ml. of 0.1 M veronal buffer of pH 9.0, 0.2 mg. enzyme and 1.0 ml. of 2% substrates. The reaction was carried out at 37°C for 10 minutes.

With heart-treatment at 100°C for 10 minutes, casein was hydrolyzed at the highest rate and the hydrolytic rate of serum albumin was only 10 per cent of that of casein. Both hemoglobin and serum albumin without heat-treatment were hydrolyzed to a less degree than with heat-treatment. A similar experiment for the liberation of trichloroacetic acid-soluble peptides from lysozyme is shown in Fig. 5.

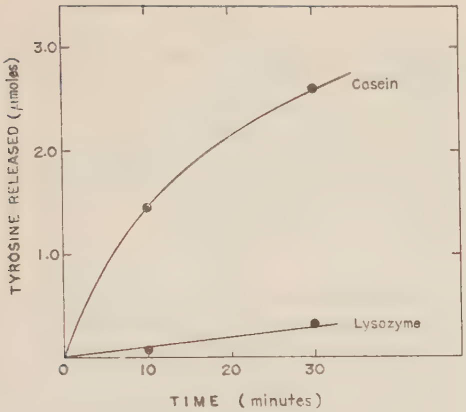

FIG. 5. Proteolytic activity of Hβ-proteinase on casein or lysozyme as substrate.
An incubation mixture contained 400 μg. of Hβ-proteinase, 10 mg. of lysozyme or casein and 25 μmoles of veronal buffer. The reaction was carried out at pH 7.35 and 37°C. Total volume, 1.0 ml.

Lysozyme was hydrolyzed to a far less extent than casein. This result was also ascertained by the alcohol-alkali titration method as seen in Table II.

Activities of Hβ-proteinase on Various Synthetic Substrates—As shown in Table III, Hβ-proteinase failed to hydrolyze all 17 synthetic substrates in the conditions described above. It is especially of interest that benzoyl-L-arginine amide, carbobenzoxy-L-glutamyl-L-tyrosine and L-tyrosine ethyl ester which are the typical synthetic substrates for trypsin, pepsin and chymotrypsin, all failed to be hydrolyzed by Hβ-proteinase.

TABLE II

Measurement of Hβ-proteinase Activity on Casein and Heat-denatured Lysozyme by the Method of Potentiometric Titration

Incubation	NaOH consumed	
	Casein	Lysozyme
min.	μmole	μmole
60	1.3	0
120	1.8	0

The reaction mixture contained 175 μg. enzyme, 5 mg. of casein or lysozyme and 8 μmoles of Na_2HPO_4. The pH was adjusted to 7.35 with NaOH. Incubation temp. 37°C. Total volume 0.25 ml.

TABLE III

Activity of Hβ-proteinase on Various Substrates

Substrate	Assay Method[1]		Activity
Bz-L-Arg-NH_2	p,	t	—
Cbz-L-Glu-L-Tyr	p		—
L-Tyr-Et	p,	t	—
Chloroacetyl-L-Tyr	p		—
p-Amino Bz-Glu	p,	t	—
Gly-L-Tyr	p,	t	—
Gly-DL-Phe	p		—
Gly-L-Leu	p		—
Gly-DL-Norleu	p		—
DL-Ala-DL-Glu	p		—
DL-Ala-L-Leu	p		—
L-Leu-Gly	p		—
L-Leu-L-Tyr	p		—
DL-Val-Gly	p		—
DL-Phe-Gly	p		—
L-Leu-Gly-Gly	p,	t	—
Gly-Gly-Gly	p,	t	—

1) p; Assay by paper chromatographic method
t; Assay by alcohol-alkali titration method

Hydrolysis of Whale Insulin by Hβ-proteinase—Hydrolysis of glycyl chain of whale insulin by Hβ-protoinase was investigated. The uptake of alkali after the complete hydrolysis of 1 μmole of glycyl chain by Hβ-proteinase was 1 μmole, as seen in Table IV. From this result it is suggested that glycyl chain was decomposed into two fragments. N-terminal amino acids of enzymatic degradates were identified as glycine and glutamic acid by two dimensional paper chromatography, as shown in Fig. 6, and as glycine is the original N-terminal residue of glycyl chain, the newly resulting N-terminal amino acid was identified as glutamic acid. For the quantitative determination, the two spots were eluted with hot water and the optical density at 360 mμ was determined for each extract. Table V shows the ratio of glycine to glutamic acid in the course of 6 *N* HCl hydrolysis. From this result it is concluded that glutamic acid resulting from the enzymatic hydrolysis is

TABLE IV

Measurement of Proteinase Activity on Glycyl Chain of Whale Insulin by the Method of Titration

Time	NaOH	NaOH/Glycyl Chain
hrs.	μmole	
19	1.76	1.10
23	5.44	0.90

The incubation mixtures with 0.25 ml. of the total volume contained 1.6 μmoles of glycyl chain, 0.4 mg. of enzyme and 8 μmoles of Na_2HPO_4. The pH was adjusted to 8.1. The reaction was carried out at 34°C and stopped by the addition of 1 ml. ethanol and titrated with 0.02 N NaOH which contained 80% ethanol.

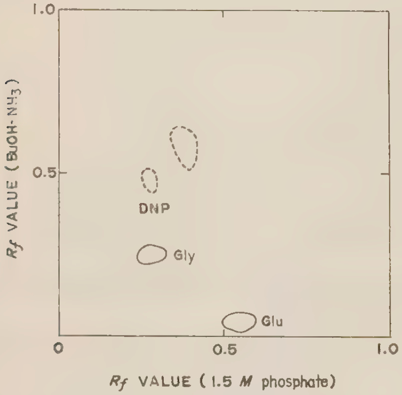

FIG. 6. Paper chromatogram of N-terminal DNP-amino acids of enzymatic degradation products of glycyl chain.

TABLE V

Change of the Ratios of Glycine to Glutamic Acid in the Course of Acid Hydrolysis

Hydrolysis Time	Ratio of Glu to Gly
hrs.	
4	1.2
12	2.8

equivalent to glycine of the N-terminal residue of glycyl chain.

Similarly, the uptake of alkali after the enzymatic hydrolysis of 1 μmole of phenylalanyl chain was found to be 3 μmoles, as seen in Table VI, suggesting that phenylalanyl chain was decomposed into four fragments.

TABLE VI

Measurement of Proteinase Activity on Phenylalanyl Chain of Whale Insulin by the Method of Titration

Time	NaOH	NaOH/Phe-chain
hrs.	μmole	
16	6.0	3.0
24	6.2	3.1

The incubation mixtures with 0.25 ml. of total volume contained 2 μmoles of phenylalanyl chain, 0.64 mg. enzyme and 8 μmoles of Na_2HPO_4. The pH was adjusted to 8.1 with NaOH. The reaction was carried out at 35°C, and stopped by the addition of 1 ml. of ethanol and titrated with 0.02 *N* NaOH containing 80% ethanol.

Fig. 7 shows that N-terminal residues of the enzymatic degradation products are phenylalanine, valine, glutamic acid and traces of unknown spots. The quantitative determination of each spot shows two moles of valine and 0.5-0.7 mole of glutamic acid per 1 mole of phenylalanyl chain.

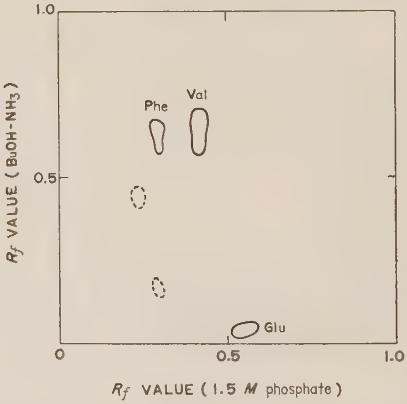

FIG. 7. Paper chromatogram of N-terminal DNP-amino acids of enzymatic degradation products of phenylalanyl chain.

Since according to Sanger *et al.* (*13*) the peptide bonds associated with three molecules of valine found in phenylalanyl chain are two leucyl valines and one phenylalanyl valine, it will be obvious from our results that leucyl valine is splitted by Hβ-proteinase.

DISCUSSION

The important symptoms of Habu bite are local pathological changes characterized by hemorrhagic necrosis, swelling and intense pain. In a series of our previous reports (*1–5*, *10*), it was suggested that Habu proteinase is one of the principles of myolysis brought about by Habu bite. Recently it was found by us that the crude Habu snake venom heated at 100°C for ten minutes still caused drastic myolysis without hemorrhage, whereas the crude venom after heat-treatment did not show any proteolytic activity on casein (*11*). From these results it is strongly suggested that there are several principles in crude Habu snake venom which cause pathological changes in muscle resulting from Habu bite.

It was found that Hβ-proteinase shows a high substrate specificity compared with S-proteinase (*14*). In the experimental conditions described above, Hβ-proteinase did not hydrolyze α-benzoyl-L-arginine amide known as a synthetic substrate for trypsin and papain, carboxy-L-glutamyl-L-tyrosine as a synthetic substrate for pepsin, and carbobenzoxy-L-tyrosine ethyl ester as a synthetic substrate for chymotrypsine. All these synthetic substrates are hydrolyzed by S-proteinase.

According to Deutsch and Diniz (*12*), all the snake venoms of *A. mokeson*, *C. atrox* and *C. horridus* hydrolyzed benzoyl-L-arginine ethyl ester and the activity, unlike that on hemoglobin, is not inhibited by ethylenediamine tetraacetic acid. They also reported that no significant hydrolysis of acetyl-L-tyrosine ethyl ester was found with the snake venoms described above. These suggested that each of the proteolytic enzymes in a snake venom has a different substrate specificity.

From the quantitative determination of N-terminal residues formed after the enzymatic hydrolysis of phenylalanyl chain of whale insulin, it will be suggested that the peptide bond of leucyl valine is splitted by Hβ-proteinase. The peptide bond in glycyl chain, from which glutamic acid is released as N-terminal amino acid after hydrolysis by Hβ-proteinase, is not yet investigated.

Normal sera obtained from horse, rabbit and man have a strong inhibitory effect on S-proteinase and especially on trypsin compared with that on Hβ-proteinase. This may be one of reasons why Hβ-proteinase causes a more serious necrosis of muscle with hemorrhage, in spite of its high substrate specificity, than that caused by trypsin and S-proteinase.

SUMMARY

Normal sera obtained from horse and rabbit show a strong inhibitory effect on S-proteinase and especially on trypsin, but their effect on Hβ-proteinase from Habu venom is far weaker. Of the proteins tested, Hβ-proteinase is able to hydrolyze casein most rapidly, but fails to hydrolyze all synthetic substrates investigated.

N-terminal amino acids resulting from the hydrolysis of whale insulin by Hβ-proteinase are glutamic acid and valine in phenylalanyl chain, and glutamic acid in glycyl chain, respectively.

The fact that Hβ-proteinase can cause serious pathological lesions in spite of its high substrate specificity may be explained partly by the less inhibitory effect of sera on Hβ-proteinase, which inhibit strongly the activity of proteinases such as trypsin and S-proteinase.

We are greatly indebted to Prof. R. Sato, of the Institute for Protein Research, Osaka University, for his invaluable advice and discussion.

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The Presence of Adenylyl 2, 3-Diphosphoglyceric Acid in Pig Blood

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The presence of a minor adenine nucleotide in human blood has been reported by may workers (*1–4*). Recently the present authors have isolated from pig blood a hitherto unknown adenine nucleotide which corresponded in composition to adenylyl diphosphoglyceric acid (*5*). The detailed study on the nucleotide is the subject of this commnication.

EXPERIMENTALS

Materials—The barium salt of 2, 3-diphosphoglyceric acid (2, 3-DPGA) was isolated from pig blood (*6*). L-Glyceric acid was prepared from 2, 3-DPGA by sulfuric acid hydrolysis (*7*), and DL-2-phosphoglyceric acid (2-PGA) was synthesized from β-glycerophosphate (*8*). Glyoxylic acid and glycolic acid were prepared from oxalic acid (*9*). The calsium salt of bis (*p*-nitrophenyl)-phosphate which was used as substrate of phosphodiesterase, was also synthesized (*10*). Other reagents were commercial. Ribose-5-phosphate (R-5-P) and 3-phosphoglyceric acid (Schwarz Laboratories Inc.) were purified by column chromatography and repeated reprecipitation as barium salts. The yielded phosphates were paper chromatographically pure, but 3-phosphoglyceric acid contained about 0.005 mole of 2-PGA per mole as checked by using enolase.

Intestinal phosphomonoesterase was commertial (Worthington Biochem. Corp.). This has a little activity of diesterase and nucleotide pyrophosphatase when examined with DPN as the substrate, glucose-glucose dehydrogenase system being used for the determination of remaining DPN (*11*). Nucleotide pyrophosphatase was prepared from snake venom (*Agkistrodon blomhoffii*) which as kindly supplied by Dr. T. Suzuki (*12*). Pure 5'-adenylic acid deaminase was prepared from rabbit skeletal muscle (*13*) and crystalline enolase from brewer's yeast (geneloulsy supplied by Kirin Brewers Co. Ltd.) (*14*). Baker's yeast crystalline phosphoglyceric acid mutase was kindly supplied by Dr. H. Chiba (*15*).

Column Chromatography—Gradient elution chromatography on Dowex 1-formate (×10) was employed for the first step separation of nucleotides (*16*). In rechromatography to separate the new nucleotide from contaminating phosphates, a slightly modified system was used. Elution was begun with water in the mixing flask and 4 *N* formic acid-0.4 *M* ammonium formate in the reservoir.

For further purification of the nucleotide and separation of each hydrolyte from its hydrolysate, stepwise elution technique on Dowex 1-Cl (X10) column with following elution sequence was employed: 0.03 *M* NH_4Cl, 0.1 *M* NH_4Cl, 0.1 *N* HCl (*17*).

For separation of of glyceric acid (*18*), Amberlite CG-400-carbonate (Type 1) column was used. Glyceric acid was eluted by 0.05 *M* ammonium carbonate at pH 9.5 and the free acid was recoverd by removing the salt through a cation exchanger Amberlite CG-120-H (Type 1). Glyceric acid was recovered 95 per cent or more by these procedures.

Paper Chromatography and Paper Electrophoresis—These method were employed for the criteria of the purity of the nucleotide and the identification of its hydrolysis products. The following developing solvent mixture were used for phosphorus compounds; A, *n*-butyric acid-0.5 *N* ammonia (*19*); B, *n*-butyric acid-NaOH (*20*); C, 0.1 *M* phosphate buffer at pH 6.8-ammonium sulfate-*n*-propyl alcohol (*21*); D, *t*-butyl alcohol-picric acid-water (*22*); E, *n*-propyl alcohol-ammonia-water (*22*), and its modification (*6*); F, formic acid-water-95% ethyl alcohol using molybdate impregnated paper (*23*). Bases were chromatographed by the following two systems; G, *i*-propyl alcohol-2 *N* HCl (*24*); H, *n*-butyl alcohol-ammoniawater (*25*).

Paper electrophoresis was employed for rapid separation subsidiarily to the paper chromatography. Pyridine-acetic acid-water mixture at pH 3.6 was used and a high voltage (100 volts per cm. length) was applied.

Paper chromatographical separation of 2, 4-dinitrophenylhydrazone derivatives was conducted by two solvents: 0.05 N NaOH and n-butyl alcohol saturated with 3 % ammonia (*26*).

The detection of the spots was made with aid of an ultraviolet light lamp (Manasuru lamp) or by spraying with molybdate reagent (*22, 27*). Radioactivation analysis (*28*) was used for the estimation of a minute quantity of phosphates on the paper by irradiation with thermoneutron flux in the Atomic Reactor JRR-1 at Japan Atomic Energy Research Institute.

Other Method—Ultraviolet absorbing compounds were determined spectrophotometrically, adopting the following molar extinction coefficients at 260 mμ in acid: adenine, 13,600; 5'-AMP, 14,600; ADP, 14,300; ATP, 14,300. That of the new nucleotide was taken as 14,300.

Phosphorous was determined semiquantitatively by the method of Horecker (*29*) in the course of preparative procedures, and more presisely by the method of Berenblum and Chain (*30*) on the purified substances.

Periodate oxidation of nucleotide was conducted spectrophotometrically (*31*), and oxidation of gryceric acid by the method of Burton (*32*).

The following color reactions were employed: orcinol reaction (*33*) for pentose, chromotropic acid reaction (*34*) for gryceric acid and its derivatives, *i. e.*, formaldehyde and glycolic acid (*32, 35*). 2, 4-Dinitrophenylhydrazone method was used for the formaldehyde (*36*) and glyoxylic acid (*37*). Anthrone (*38*) and ninhydrin (*39*) reaction were also employed.

Enzymatic determinations were also used and these procedures are shown in respective experiments.

RESULTS

Preparative Procedure—5 liters of oxalated pig blood (potassium oxalate 2 mg./ml.) was extracted with 10 liters of ice-cold perchloric acid (0.6 N), and filtered through a diatomaceous earth pad (Johns-Manville, Celite) with suction. During further operations the solutions containing the new nucleotide were kept in the ice-cold. The filtrate was neutralized to phenolphthalein with NaOH and phosphates were precipitated by addition of sufficient amount of 25 % barium acetate (100 ml.). About 20 g. of barium salt was also obtained from 5 liters of the blood. The barium salt which contained about 1.2 m mole of adenine nucleotides was dissolved in one or two liters of 0.2 N HCl and any insoluble material removed by filtration. The clear filtrate was run through a column of charcoal (50 g. of charcoal, Shirasagi A, Takeda) which had been previously treated with octyl alcohol (*40*). The column was washed successively with two column volumes of 0.2 N HCl and water. The percolates and washing were combined and the unadsorbed (non-nucleotid) phosphorous compounds were precipitated as barium salt. Nucleotides adsorbed on the charcoal column were eluted with 50 % ethyl alcohol in 0.2 N ammonia, and precipitated by addition of sufficient amount of barium acetate and an equal volume of ethyl alcohol. A perfect separation of nucleotides from non-nucleotide phosphates could not be accomplished probably due to the large scale fractionation. In one of the experiments, barium salt of the non-nucleotide fraction weighed 6 g. and that of the nucleotide fraction 3 g. which contained about 0.9 mmole of nucleotides (yield: 75 per cent).

Isolation Procedure—The barium salt of the nucleotide fraction was the subject to the first column chromatographic analysis. After removal of barium with Amberlite IR 120-H, the nucleotide was chromatographed

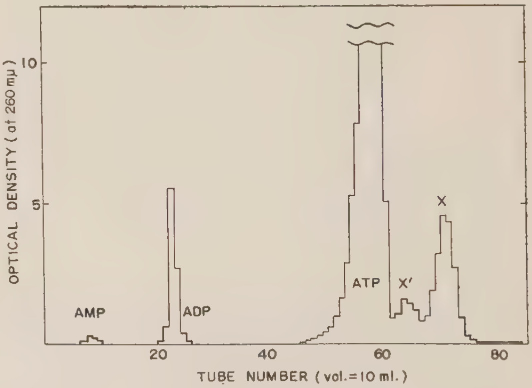


FIG. 1. Rechromatography by the modified formic acid system of the ATP plus X fraction.

The nucleotides were freed from barium with cation exchange resin, and chromatographed on Dowex 1-formate column (1×20 cm.). Mixer volume was adopted 300 ml. for convenience, and elution was started directly with 4 N formic acid-0.4 M ammonium formate in the reservior. Peak X is the new nucleotide fraction.

on a column of Dowex 1-formate (*16*). A minor peak of an unknown adenine cucleotide was eluted just after ATP (*5*). This peak was separated together with the tail of ATP peak from the succeeding peaks of other compounds such as guanosine triphosphate, uridine triphosphate and contaminant 2, 3-DPGA. The nucleotides were adsorbed on a charcoal column, eluted ammonia alcohol and directly run through a column of Dowex 1-Cl. After the column was completely washed with 0.1 M NH_4Cl at pH 5.0, the nucleotide were eluted by 0.1 N HCl.

To remove the contaminating ATP, the nucleotides were rechromatographed on a column of Dowex 1-formate with gradient elution from water to 4 M formic acid-0.4 M ammonium formate (Fig. 1). The peak of the nucleotide (X) was separated from the peaks of ATP and an unknown nucleotide (X') which was present occasionally in the nucleotide precipitate. The solution was again treated with charcoal and Dowex 1-Cl column. The isolated nucleotide was precipitated as barium salt. For further examinations, it was decationized by treating with cation exchanger.

The purity of the material was estimated by paper chromatography (Solvent A) followed by activation with thermoneutron irradiation. Approximately 98 per cent of the activated phosphorous was located at the position of the new nucleotide, and the rest was detected at the positions corresponding to 2, 3-DPGA and AMP.

Pure crystals of its sodium salt was obtained from the solution neutralyzed with NaOH by addition of three volumes of ethyl alcohol at 0°C, the gummy precipitate being converted to fine and very deliquescent needles upon washing with ethyl alcohol.

Components of the New Nucleotide—This material gave ultraviolet spectra at different pH's characteristic to adenosine phosphate as presented in Fig. 2. But it behaved quite differently from ATP on paper chromatography when run in four different solvents-A, B, C, and E (Fig. 3). Its R_f values were close to those of ATP in some solvents, but satisfactory separation of both nucleotides could be effected in solvent B.

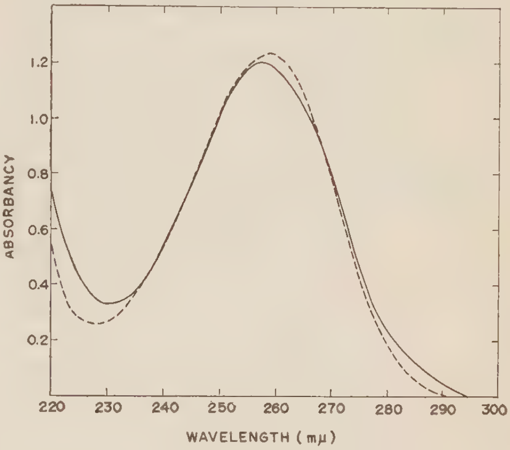


Fig. 2. Ultraviolet absorption spectra of the nucleotide.

The spectra are idential to that of adenine nucleotide in acid (—) and in neutral solution (---).

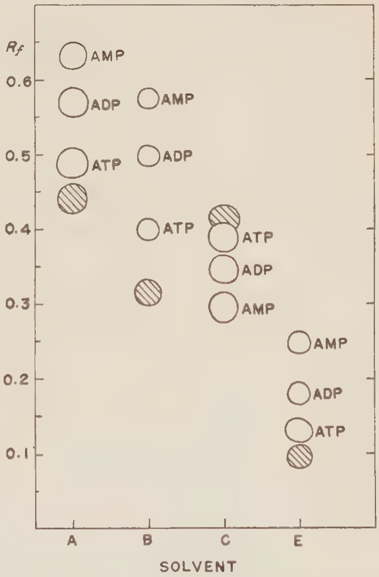


Fig. 3. Schematic paper chromatogram of the new nucleotide and other adenine nucleotides. The hatched circles indicate the location of the nucleotide in question.

A) *n*-butylic acid-NH_4OH
B) *n*-butylic acid-NaOH
C) phosphate buffer-$(NH_4)_2SO_4$-*n*-propyl alcohol
E) *n*-propyl alcohol-ammonia-water

By acid hydrolysis, it readily liberated the base which was identified as adenine by spectrophotometry and paper chramotography (*24*, *25*).

The presence of ribose as a component was indicated by the chracteristic orcinol reaction. The absence of other sugers was demonstrated by the fact that the color develpment and its density by anthrone reaction (*38*) could be fully explained by the ribose content. Periodate was consumed equivalent mole to the nucleotide and the oxidation caused the disappearance of the orcinol reaction. This observation showed the presense of vicinal hydroxy groups of ribose moiety.

The characteristic property of the compound was the blue-green color in the chromotropic acid reaction. The spectrum was very similar to that described by Bartlett (*43*) for 2, 3-DPGA as shown in Fig. 4A. Moreover the color developed at the same rate during heating both compounds with the reagent (Fig. 4B).

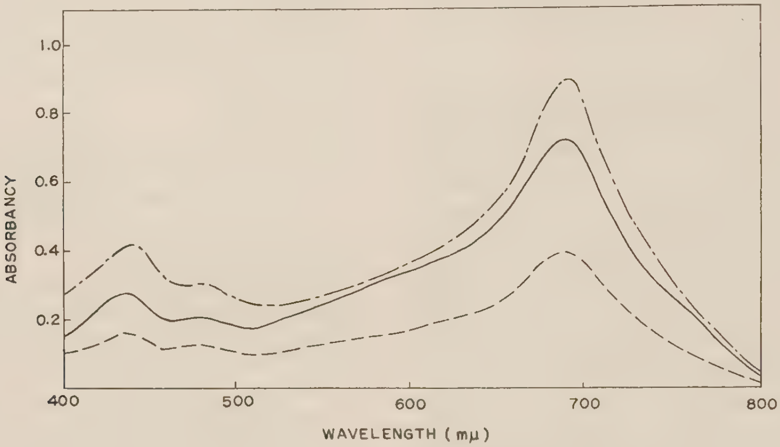


Fig. 4A. Spectra of the chromotropic acid reaction.
—-—, ——, ----: See Fig. 4B.

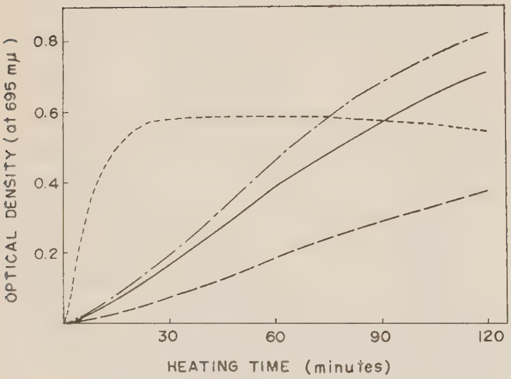


FIG. 4B. Difference of color development of samples.

The nucleotide was whole (—-—) and its 2, 3-DPGA component (---) isolated from the nucleotide hydrolysate, and authentic 2, 3-DPGA (——) were used to the chromotropic acid reaction.

Color developments of these compounds are apparentrly different from that of 3-PGA (····). A little high absorbancy of the nucleotide at 400-500 mμ region is due to the ribose moiety.

The ninhydrin reaction (*39*) was negative even when a sufficient amount of the nucleotide was tested.

A free acid preparation of the new nucleotide was subjected to analysis simultaneously with ATP in the same run. The results are given in Table I.

Products of Hydrolysis—Hydrolysis of the nucleotide in *N* HCl at 100°C for 10 minutes yielded an approximately equimolar mixture of adenine, R-5-P and 2, 3-DPGA which were separated by column chromatography (Fig. 5). In addition, a small amount of inorganic phosphate was released. R-5-P was identified by paper chromatography and by the determination of the ratio of ribose to phosphate. The color reaction for glycerate was positive only

TABLE I

Analyses of the New Nucleotide Compared with ATP

	Adenine	Ribose	Labile P	Total P	Glyceric acid
ATP	1.00	1.01	1.88	2.94	—
New nucleotide	1.00	0.98	0.15	3.04	0.99

Analytical values are expressed as moles per mole of adenine. The molar extinction coefficient of adenine nucleotides at 260 mμ in acid was taken as 14,300. Ribose was determined by the orcinol reaction (*33*) using AMP as a standard, phosphorous by the method of Berenblum and Chain (*30*), and glyceric acid by the chromotropic acid reaction (*34*) using 2, 3-diphosphoglyceric acid as a standard.

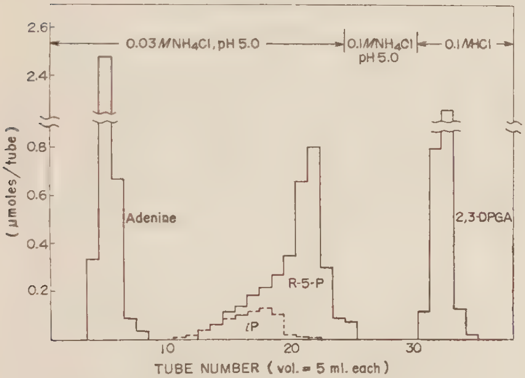


Fig. 5. Dowex 1-chloride chromatography of the acid-hydrolysate of the nucleotide.

3.5 μmoles of the nucteotide was hydrolyzed in *N* HCl at 100°C for 10 minutes. The mixture was dried *in vacuo* at room temperature to expel HCl, and applied to Dowex 1-Cl column (1 × 1cm).

Equal amounts of adenine (3.55 μmoles), ribose (3.35 μmoles), 2, 3-DPGA (3.51 μmoles) and small amount of inorganic phosphate (0.67 μmole) were recovered in the eluates.

in the eluate with 0.1 *N* HCl but negative in the eluate with 0.1 *M* NH_4Cl which would readily eluate monophosphoglyceric acid.

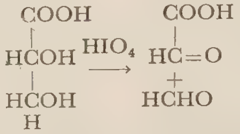
Identification of 2, 3-DPGA—2, 3-DPGA in the hydrolysate was identified by several methods as follows. It was located at the same position as the authentic sample of 2, 3-DPGA upon paper chromatography and gave the expected molar ratio of glyceric acid to phosphate 1 : 2 by analysis. To obtain the organic component, the material was submitted to hydrolysis by intestinal phosphomonoesterase. 0.1 ml. of the enzyme solution (20 units) was added to the mixture of 0.1 ml. of 2, 3-DPGA fraction (2 μmoles) and 0.1 ml. of ethanolamine-HCl buffer at pH 9.5 and incubated at 38°C for 30 minutes. The reaction mixture was diluted with water and passed through an Amberlite CG-400-carbonate column (0.5 × 2cm.). The column was washed with several column volumes of water. Glyceric acid was eluted with 0.05 *M* ammonium carbonate at pH 9.5 where inorganic phosphate was retained in the column. Mono- and diphosphoglyceric acid could not be detected by chromatographic analysis, and the organic component was nearly quantitatively recovered as glyceric acid. The solution was passed through an Amberlite CG-120-H column for the purpose of complete removal of ammonium ion, and concentrated *in vacuo* to expel carbon dioxide. The remaining acid was not extractable with ether and not volatile. Its periodate oxidation products (*32*), formaldehyhe and glyoxylic acid, were detected as the 2, 4-dinitrophenylhydrazone derivatives as follows, and by the chromotropic acid color reactions. The yielded neutral and acid hydrazones coincided with hydrazones of formaldehyde and glyoxylic acid, respectively, in their spectra as well as R_f values on paper chromatography. A portion of the periodate oxidation mixture was taken for the determination of formaldehyde. Another portion was evaporated to expel formaldehyde, remaining glyoxylic acid reduced to glycolic acid by the addition of magnesium powder and sulfuric acid and determined by its red color by the chromotropic acid reaction (*35*). The analytic values, as given in Table II, are in agreement with those obtained from an authentic sample of glyceric acid.

Final proof for 2, 3-DPGA was provided by its activating effect on the phosphoglyceric acid mutase reaction. In a quartz absorption cell of 1 cm. path placed 1 ml. of the same buffer adopted by Warburg and Christian (*14*) except that the DL-2-PGA was reduced to 2.5 μmoles per ml. and in

TABLE II

Analyses of Glycerate Obtained from the New Nucleotide Compared to the Authentic

Glyceric acid was used 0.065 and 0.77 μmole respectively (determined by the chromotropic acid reaction). Experimental procedures are shown in text. All values are expressed as μmole.



$$\begin{array}{c}COOH\\ |\\ HCOH\\ |\\ HCOH\\ H\end{array} \xrightarrow{HIO_4} \begin{array}{c}COOH\\ |\\ HC{=}O\\ +\\ HCHO\end{array}$$

Methods / Sample	Chromotropic acid reaction	2, 4-Dinitrophenylhydrazone		Chromotropic acid reaction	
		Formaldehyde	Glyoxylic acid	Formaldehyde	Glyoxylic acid
Authentic glycerate	0.065	0.068	0.065	0.063	0.063
Glycerate fraction of the hydrolysate	0.077	0.078	0.077	0.080	0.082

another cell, water as the blank. The optical density at 240 mμ versus the blank was measured. To each cell, bank included, were added 20 μg. of enolase and 15 μg. phosphoglyceric acid mutase in a volume of 0.02 ml. The cell contents were mixed and kept at room temperature untill the equilibrium had been reached. 0.1 ml. of 2, 3-DPGA solution was added to each cell and the optical density measured versus the blank cell at 240 mμ was recorded at 30 second intervals. Using this system, the apparent Michaelis constant for 2, 3-DPGA was 6.5×10^{-6} *M*. This value was nearly equal to that obtained by Sutherland *et al.* (*41*), and different from those obtained by other workers (*15*, *42*). This might be due to the difference of the assay system and not to be the difference of the enzyme. The maximal effect was obtained at 2×10^{-5} *M* of both the authentic 2, 3-DPGA and the sample derived from the nucleotide. The unhydrolyzed nucleotide did not exhibit this coenzyme activity.

Presence of Pyrophosphate Bond and Identification of 5'-AMP—Lyophilyzation of the nucleotide solution in 0.1 *N* HCl yielded AMP but not ADP, whereas ADP as well as AMP was liberated from ATP by the same procedures.

Nucleotide pyrophosphatatase obtained from snake venom (*12*) was used to demonstrate the presense of pyrophosphate bond. About 0.03 unit of the enzyme (20 μg. protein) in 0.1 ml. was added to the mixture of 0.1 ml. of the nucleotide solution (0.8 μmole), 0.1 ml. of 0.2 *M* veronal-sodium acetate buffer at pH 9.0 and 0.03 ml. of 0.3 *M* $MgSO_4$. The reaction mixture was incubated at room temperature for 60 minutes. Five ml. of

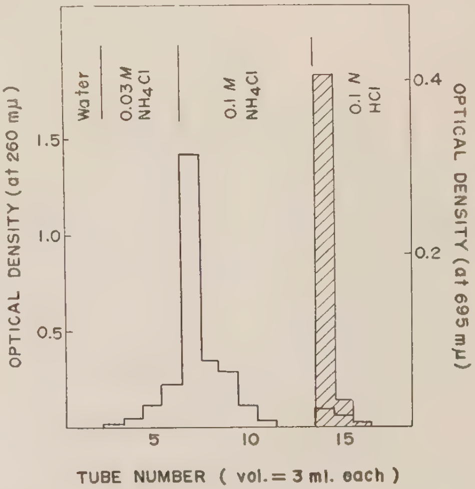


FIG. 6. Stepwise separation of products by venom nucleotide pyrophosphatase.

Open columns represent optical density at 260 mμ and hatched column represents at 695 mμ. Major peak of the nucleotide was identified as 5'-AMP by muscle deaminase and glycerate was only found in 2, 3-DPGA fraction. AMP and 2, 3-DPGA were recovered almost equal amount of the applied nucleotide.

water was added and immediately run through a column of Dowex 1-Cl (0.4×1.5 cm.), and chromatographed. The nucleotide was nearly quantitatively split into AMP and 2, 3-DPGA (Fig. 6).

The obtained AMP was identified by paper chromatography, paper electrophoresis, periodate oxidation and orcinol reaction. Further characterization was made by its reactivity to muscle 5'-adenylate deaminase. The deaminase (0.03 unit) was added to the reaction mixture as described by Nikiforuk and Colowick (*13*) adopting the citric acid-NaOH buffer system. AMP from the nucleotide was readily deaminated to IMP, as examined by the change in ultraviolet absorption spectrum.

DISCUSSION

The above observations lead us to the finding of one of new nucleotides as adenylyl 2, 3-diphosphoglyceric acid with having a pyrophosphate bridge. But it is not yet clarified which one of two phosphoric acid radicals in the 2, 3-DPGA is linked to phosphate of AMP.

The content of the nucleotide in pig blood was relatively constant. It was a little less than 4 per cent of the total nucleotides in one lot of the crude barium salt, and 3.5 per cent in the other. The location of this compound in the chromatogram with Dowex-1-formate column agreed with that of the unknown adenine nucleotide reported by other workers in human blood (*2*, *3*). In later experiments, we tried to prove the presence of the new nucleotide in human blood by using the same procedures described in this paper, and verified that the unknown nucleotide in human blood nothing but adenylyl 2, 3-diphosphoglyceric acid, as will be reported elswhere.

SUMMARY

1. A new adenine nucleotide was isolated from pig blood.

2. Spectrophotometric, chromatographic, chemical and enzymatic studies indicate that it is adenylyl 2, 3-diphosphoglyceric acid.

The auther wishes to thank Prof. H. Yoshikawa for his kind helpfulness and encouragement and to Dr. M. Nakao, Dr. M. Tatibana and Dr. Y. Ishii for their helpfulness and discussions throughout the period of this work. The auther also wishes to thank the member of the Department of Biochemistry, Faculty of Medicine, Gunma University, for their kind helpfulness for the preparation of some portion of the nucleotide, to Dr. H. Chiba for a kind supply of crystalline phosphoglyceric acid mutase and Dr. T. Suzuki for his snake venom giver.

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Studies on Hemoglobin

VI. Amino Acid Compositions of the Fractionated Bovine Globin α and β

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(Received for publication, June 1, 1961)

There has been a number of reports on the amino acid composition of whole hemoglobin molecules obtained from human (*1*, *2*), horse (*3*), bovine (*4*) and sheep (*5*) erythrocytes. The conjugated protein, however, possesses pairs of two different polypeptide chains, α and β*. Recently, there appeared several methods for the resolution of both chains (*6—10*) which associated together in hemoglobin molecule by their non-covalent linkage(s) (*7*, *8*). So it is hoped that the analyses will be also applied on the fractionated globin α and β.

As already reported by the present author in her previous paper (*7*), the valyl (α) and the methionyl (β) chains of bovine hemoglobin could be fractionally eluted from a column of Amberlite CG-50 of H-phase with the use of urea in N formic acid, as the effluent. In this paper will be described the amino acid compositions of the both chains so resolved.

EXPERIMENTALS

Materials—Bovine globin α and β used in this study were the same preparations as obtained in the previous paper (*7*). As described already (*7*) the both preparations were proved to be free from each other by 1-fluoro-2, 4-dinitrobenzene determination of their N-terminal group (Table I) and by their paper electrophoretic patterns (*8*).

Acetyl globin α and β were obtained from the corresponding globin fractions mentioned above, by the acetylation with acetic anhydride in the presence of concentrated urea, followed by dialysis and lyophilisation. In a typical case, 500 mg. of globin fraction were dissolved into 10 ml. of N formic acid containing urea at a concentration of 8 M*, the pH was adjusted to 7 with 2 N sodium hydroxide, and the viscous solution was further added with excess amounts (10 g.) of urea. To the mixture was added 0.5 ml. of acetic anhydride (more than 10 mole per mole of free amino group) dropwise, under a vigorous stirring for one hour during which the temperature was kept at 0° to 5°C and the pH was maintained to 7 by the additon of 2 N sodium hydroxide. After stirring at 0° to 5°C for the additional two hours, the reaction mixture (solid urea still remaining) was dialyzed in Casing sac against running tap water for two days and then against to distilled water for one day, and the content was lyophilized. The analytical data on the amino contents of intact and acetylated globin fractions are summarized in Table I.

TABLE I

α-and ε-Amino Contents of Globin Fractions[1]

Globin Fraction	α-Amino mole/mole		ε-Amino mole/mole
Globin α	Valine	1.0	10.0[2]
Acetylated globin α	less than	0.02	less than 0.2
Globin β	Methionine	1.0	11.0[2]
Acetylated globin β	less than	0.02	less than 0.2

1) The usual 1-fluoro-2, 4-dinitrobenzene method was used.

2) Presumed from the lysine content (Table II).

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* As has been demonstrated (*8*, *9*), the protein of hemoglobin seems to represent an aggregate of four polypeptide chains, α, α', β and β'. Barring the discovery of any contrary evidence. α and α' or β and β' may be assumed to be very similar if not identical.

* Dissolving globin into this solvent completely seemed to be essential for the quantitative acetylation of free amino group.

Analytical Methods—Acid hydrolysate of globin was obtained from 10 to 30 mg. portion of protein, by heating with twice redistilled 5.7 *N* hydrochloric acid (5 ml.) in a sealed tube at 110°C for 36 hours, and by repeated evaporation of the resulting solution over sodium hydroxide.

Each amino acid content in the acid hydrolysate was analyzed by the following two different methods, and was expressed as mole per mole of protein.

Method A—A portion of the residues of acid hydrolysate (3 to 5 mg.) was dinitrophenylated with 1-fluoro-2, 4-dinitrobenzene, and the resulting ether-soluble fraction, after 2,4-dinitrophenol being removed by sublimation, was resolved by two dimensional paper chromatography. 1-Butanol saturated with 5% ammonia, and 1.5 *M* sodium phosphate buffer, pH 6, were the solvent systems. The paper chromatogram is shown in Fig. 1.

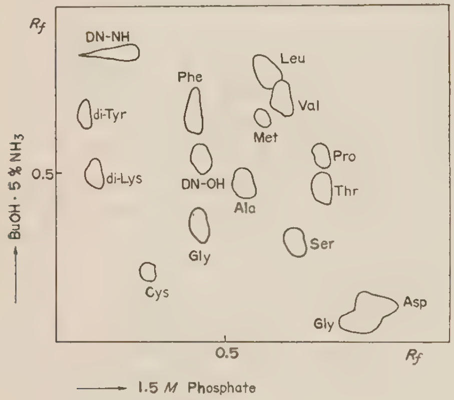

Fig. 1. Paper chromatogram of dinitrophenyl amino acids derived from globin *α*.

Solvent systems: 1-Butanol saturated with 5% ammonia and 1.5 *M* sodium phosphate buffer, pH 6.

The amount of amino acid was calculated from the optical density at 360 mμ (380 mμ was used in the case of dinitrophenyl proline) of 1% sodium bicarbonate-extract of each yellow spot and from molar extinction coefficient of the corresponding authentic dinitrophenyl amino acid in the same solvent. No correction was made to the recoveries of dinitrophenylation and of extraction from paper (*11*, *12*).

Method B—Another portion of acid-hydrolysate was analyzed, according to the TNBS*-method of Satake (*13—15*). Thus the amino acid mixture was resolved two-dimensionally on a filter paper sheet (Toyo Roshi No. 50) of 40×40 cm. by electrophoresis (pyridine-acetic acid-water; 6 : 0.5 : 94 v/v. pH 6.65, 30 volt. per cm., 60 minutes) and then by chromatogrophy (1-butanol-acetic acid-water; 5 : 1 : 5 v/v).

Each spot of amino acid was detected by trinitrophenylation with TNBS, and was determined spectrophotometrically as trinitrophenyl-amino acid. The map of amino acids is illustrated in Fig. 2. The present author should express her sincere thanks to Mr. T. Shinoda for this analysis.

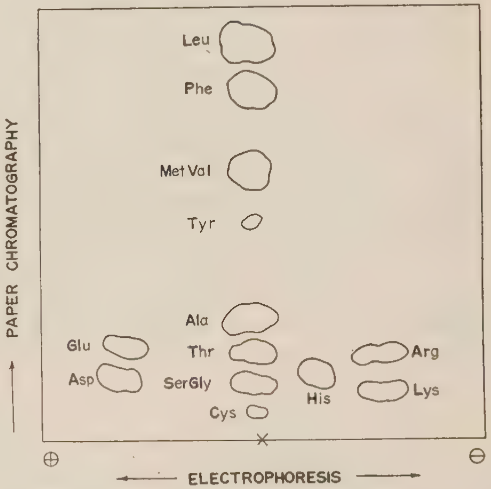

Fig. 2. Two-dimensional resolution of amino acids derived from globin *β* by electrophoresis and chromatography.

Electrophoresis: Pyridine-acetic acid-water; 6 : 0.5 : 94 v/v. pH 6.65, 30 volt. per cm., 60 min.

Chromatography: 1-butanol-acetic acid-water; 5 : 1 : 5 v/v

Coloration: Trinitrophenylation with 0.1% TNBS at pH 8.

Lysine- and arginine-contents were also estimated by comparing the amounts of N-terminals newly liberated from intact protein (N-terminals from the both of lysyl and arginyl linkages) and from acetylated protein (N-terminals only from arginyl linkages) under the action of trypsin (*16*). Tryptic hydrolysis was performed in *M*/10 sodium borate buffer at pH 8.5 and at 37°C for 6 hours, using the enzyme/substrate ratio of 1/50. Twice crystallized salt-free trypsin from Worthington Biochemical Corporation was used after the preceding acid-treatment, according to the method of Anfinsen (*17*). The pretreatment, as shown in Fig. 3, seemed to be effective for eliminations of other proteolytic activity that might contaminate in this enzyme preparation. The kind and amounts of N-terminals in tryptic hydrolysate were revealed by the 1-fluoro-2, 4-dinitrobenzene method.

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* TNBS is an abbreviation for 2, 4, 6-trinitrobenzene 1-sulfonic acid.

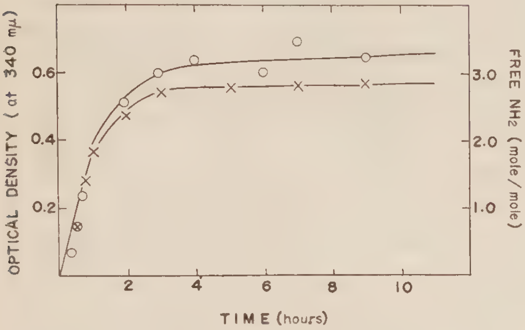

Fig. 3. Effect of pre-treatment of trypsin on the hydrolysis of acetylated bovine globin α.
—○— Trypsin before N/16 HCl-treatment
—×— Trypsin after N/16 HCl-treatment
The rate of hydrolysis was determined by the TNBS method of Satake (*14*).

Tryptophan- and tyrosine-contents were calculated from the ultraviolet absorption spectra in a range between 225 and 330 mμ of intact globin fractions in 8 M urea solution at pH 5 and pH 13 (at a concentration of one mg. per ml.) (*18*).

RESULTS AND DISCUSSION

Ultraviolet Absorption Spectra of Bovine Globin α and β—Ultraviolet absorption spectra in 8 M urea at neutral pH (presence of a shoulder at 290 mμ) indicated the presence of tryptophan residue(s) both in bovine globin α and β. The presence of tyrosine residue(s) in both globin were also suggested from a characteristic shift of absorption spectra to longer wave length by making the solution to alkaline (pH 13). By comparing the both spectra in neutral and alkaline solutions, the tyrosine- and tryptophan-contents were estimated to be 3.1 and 1.1 moles per 1.37×10^4g. of globin α, and to be 2.3 and 2.1 moles per 1.64×10^4 g. of globin β, respectively. These minimum molecular weights so estimated were in good agreement with those calculated from their N-terminal contents (one valine per 1.4×10^4g. of α, and one methione per 1.6×10^4g. of β) (Tale I).

Amino Acid Compositions of Bovine Globin α and β—Each amino acid content (except tryptophan) was estimated on 36 hours-acid hydrolysates of both globins, with the uses of two different methods, A and B. The method A, consisting dinitrophenylation of amino acids, and two-dimensional resolution and spectrophotometry of these yellow spots, permitted the assay of glycine, alanine, serine, threonine, proline phenylalanine and methionine, together with the assay of sums of valine and leucine*,** and of aspartic and glutamic acids (Fig. 1). As far as these amino acid contents were concerned, results that were reproducible to 5 per cent were readily obtained (*4*).

The method B, consisting direct resolution of acid hydrolysate, and detection and assay of each spot with TNBS, was far simple than other methods already reported. Almost every amino acid content was able to be determined by this method with an accuracy as much as 3 per cent (*15*). The only defect of this method was no reaction of proline with TNBS, and was incomplete resolutions of glycine and serine, and of valine and methionine, under the conditions used (Fig. 2). The results obtained with the uses of method A and B were in good agreement, and all amino acid contents in acid-hydrolysate could be estimated by comparing the both results.

The results, as summarized in Table II, were in approximate agreement with those reported by Craig *et al.* (*9*) on human globin α and β, both in the composition and in the minimum molecular weight. In comparison with human globin α, the corresponding bovine one (Valylleucyl chain (*7*, *8*)) contained

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* The absence of isoleucine was verified by one dimensional paper chromatography multi-developed with a freshly prepared mixture of 1-butanol, acetic acid and water (4: 1: 5 v/v) according to the method of Block (*19*).

** Incomplete resolution of DNP-valine and -leucine may be due to presence of acid-resistant peptide such as valylleucine between the both spots, even after 36 hours-hydrolysis.

TABLE II

Amino Acid Compositions of Bovine Globin α and β

(Amino acid contents are expressed as mole per mole)

Amino Acid	Globin α				Globin β			
	Bovine			Human	Bovine			Human
	Method A	Method B	Near to Integral		Method A	Method B	Near to integral	
Gly	10.0	19.1	10	6	11.8	19.2	12	13
Ser	9.3		9	9	7.1		7	5
Ala	18.2	17.4	18	19	19.0	19.6	19	14
Met	0.9	10.9	1	3	1.8	18.4	2	1
Val	26.3		10	12	35.1		16	18
Leu		16.1	16	16		19.3	19	19
Ileu			0	0			0	0
Thr	7.3	7.2	7	8	7.9	7.7	8	7
Pro	4.6	—	5	7	7.1	—	7	7
Phe	8.3	7.5	8	7	8.3	7.8	8	8
Tyr	1.9	2.6	3	3	2.0	1.8	2	3
Try[1]	— (1.1)	—	1	1	— (2.1)	—	2	3
Glu	17.6	7.0	7	5	25.3	10.1	10	10
Asp		11.3	11	11		15.1	15	13
CysH	0.5	1.0	1	1	0.6	0.9	1	2
Arg	—	3.0	3	3	—	4.0	4	4
Lys	—	10.3	10	10	—	10.9	11	10
His	—	7.0	7	10	—	7.1	7	9
Total			127	131			150	146
Molecular Weight			13,700	14,400			16,400	16,300

1) Tryptophan content was calculated from the ultraviolet absorption spectra of globin in acid and in alkaline solutions.

much more glycine (10/6)*, phenylalanine (8/7) and glutamic acid (7/5) but somewhat less histidine (7/10), proline (5/7), methionine (1/3), valine (10/12) and threonine (7/8). As regards globin β, there seemed also to be a little differences between their polypeptide chains. Thus bovine one was rich in alanine (19/14), methionine (2/1), threonine (8/7), lysine (11/10), and serine (7/5) and aspartic acid (15/13), but less in histidine (7/9), valine (16/18), tryptophan (2/3), tyrosine (2/3), cysteine (1/2)** and glycine (12/13).

Genetically determined differences in glycine-, alanine-, varine-, leucine-, serine- or threonine-content, have been considered to be common for protein molecules (*20*, *21*), and those in cysteine- (*22*) and in histidine- (*1—5*) contents have been also pointed out on whole hemoglobin molecules by several investigators. The differences in tryptophan- and tyrosine- contents in globin β elucidated by the present author, seemed to be interesting. Recently, Jollès *et al.* (*23*) also reported

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* Parenthetical values are numbers of amino acid residue present in bovine (upper) and human (lower) globins.

** No disulfide linkages had been reported on hemoglobin.

TABLE III

N-Terminals Liberated with Trypsin from Intact and Acetylated Globins[1]

Amino Acid	Globin α		Globin β	
	Intact	Acetylated	Intact	Acetylated
	mole/mole	mole/mole	mole/mole	mole/mole
Gly	3.2 (3)	0	3.1 (3)	0
Ala	2.1 (2)	0	4.2 (4)	1.1 (1)
Val	4.2 (4)	2.1 (2)	3.0 (3)	0
Leu	1.0 (1)	0	2.9 (3)	1.0 (1)
Ser	1.0 (1)	0	0	0
Thr	0.9 (1)	0	0	0
Phe	0.8 (1)	0.9 (1)	1.0 (1)	1.0 (1)
Met	0	0	1.0 (1)	0
Glu	0	0	1.1 (1)	1.1 (1)
Total	$13-1=12$[2]	3	$16-1=15$[2]	4
Lys≒12−3=9		Arg=3	Lys=15−4=11	Arg=4

1) The usual 1-fluoro-2, 4-dinitrobenzene method was used.

2) The α chain contains one mole of valine as the original N-terminal; and β, one mole of methionine. These values are subtracted.

similar genetically determined differences among lysozymes obtained from several sources, and Hagiwara* noticed some differences of absorption spectra (in a range between 260 and 300 mμ) among various crystalline cytochrome c.

Together with these differences in amino acid compositions between human and bovine globins, it should be emphasized here that their leucine-, and arginine-contents were very close to each other, either in α or in β chain.

Lysine and Arginine Residues in Bovine Globin α and β—As shown in Table II, bovine globin α seemed to contain three arginine and ten lysine residues, while the β four arginine and eleven lysine residues. These results were further verified by comparing the amounts of N-terminals liberated with trypsin from intact and from acetylated globins. As trypsin has been considered to possess a substrate specificity acting only on lysyl (not on ε-acetyllysyl) and arginyl linkages (*16*), so the N-terminals liberated from intact protein but not from the acetylated one, should be the amino acid residues following to lysyl linkage. The N-terminals liberated even after completely blocking the ε-amino groups with acetyl (see Table I), in turn, will be derived from arginyl linkage with trypsin, if the proteolytic activities due to the contaminant enzymes are negligible* (see Fig. 3).

The results obtained, as summarized in Table III, suggested the presence of three arginine residues, Arg-Val, Arg-Val and Arg-Phe in α and of four arginine residues, Arg-Glu, Arg-Ala, Arg-Leu and Arg-Phe in β, respectively. The suggestion was in good agreement with the results shown in Table I, and with the results of Satake who isolated three different arginine peptides, Ala-Arg, Leu-Arg and Thr-GluNH$_2$-Arg from bovine globin α, and four different arginine peptides, Asp-Val-Arg, Gly-Arg, Ala-His-His-Arg Glu-(Val, Leu)-Arg from bovine globin β under the action of a mixture of trypsin and chymotrypsin, respectively*.

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* Hagiwara, B., private communication.

* Recently, Kotaki in this laboratory found that the same trypsin preparation (pre-treatment being omitted) catalyzed the hydrolysis of oxidized insulin B, not only at the positions of Glu-Arg and Lys-Ala, but also at the position of Leu-Tyr was confirmed to be due to the action of trypsin itself (cross-reactivity of trypsin and chymotrypsin) (*24*).

The results shown in Table III also suggested the presence of nine lysine residues in globin α and eleven in globin β. The latter values was in good agreement with the results of direct amino acid analysis (Table II); while the former was one less (nine instead of ten). This tryptic hydrolysis method, however, had defects that the C-terminal lysine and arginine, and probably lysyl- and arginyl-proline, too, if it was present, escaped from the detection. Recently Ando *et al.* found a liberation of basic amino acid(s)** from hemoglobin under the action of carboxypeptidase B, and Satake *et al.* found that bovine globin α was resistant against carboxypeptidase A, differing from the β which liberated histidine, tyrosine and small amount of varine under the same conditions (See also *26*, *27*). Thus bovine globin α might contain more lysine*** than those (9 residues) as shown in Table III.

The absence of lysyl- or arginyl-prolyl linkage(s) in bovine globin has not been elucidated yet. The dinitrophenyl peptides derived from bovine globins by tryptic hydrolysis and by dinitrophenylation, on acid hydrolysis, gave no dinitrophenol (a main decomposition product of dinitrophenylprolyl with acid) indicating the absence of appreciable amounts of proline at their N-terminals. These results, however, will not exclude the presence of lysyl- or arginyl-prolyl linkage(s) completely, as the linkage might be too resistant against trypsin-catalyzed hydrolysis to produce prolyl terminal in such an appreciable amount under the conditions used (for 6 hours at 37°C with the use of enzyme/substrate ratio of 1/50).

SUMMARY

1. The amino acid compositions of bovine globin α and β were determined by two different methods: (a) dinitrophenylation of amino acids, and resolution and spectrophotometry of these yellow spots; (b) direct resolution of amino acids, and assay as their trinitrophenyl derivatives.

2. Bovine globin α and β had the following composition:

α—Gly 10, Ala 18, Val 10, Leu 16, Ileu 0, Pro 5, Phe 8, Tyr 3, Try 1, Ser 9, Thr 7, CysH 1, Met 1, Arg 3, His 7, Lys 10, Asp 11, Glu 7, Total 127, molecular weight 13,700.

β—Gly 12, Ala 19, Val 16, Leu 19, Ileu 0, Pro 7, Phe 8, Tyr 2, Try 2, Ser 7, Thr 8, CysH 1, Met 2, Arg 5. His 7, Lys 11, Asp 15, Glu 10 Total 150, molecular weight 16,400.

3. By comparing the N-terminals liberated from intact and from completely acetylated bovine globin, the arginyl and lysyl residues were presumed to present as the following sequences.

α—Arg- Val, -Val, -Phe.
Lys- Gly (3), -Ser, -Thr, -Ala (2), -Val, -Leu, His-Lys-OH.
β—Arg- Glu, -Ala, -Leu, -Phe.
Lys-Gly (3), -Ala (3), -Val (3), -Leu (2).

The author wishes to express her gratitude to Prof. K. Satake for his interest and discussion, and also to Mr. Y. Kimura, Miss M. Yoshida and Miss Y. Nagatsuka for their assistance in the experiments.

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* Satake *et al.* (*25*) had already proved that the arginine residues in bovine hemoglobin (14 residues per 68,000 g.) presented as pairs of these seven different linkages.

** Ando, T., and Kawanishi, Y., (private communication).

*** Recently the C-terminal structure of bovine globin α was found to be—His-Lys-OH, while that of human globin α to be—Tyr-Arg-OH (Satake *et al*, unpublished).

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Stero-Bile Acids and Bile Sterols

XL. Biosynthesis of Pentahydroxycoprostane

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(Received for publication, January 19, 1961)

Generally speaking the amphibian bile contains neutral sterols as were isolated from toad and bull frog bile by Kazuno *et al.* and named bile sterols (*1–3*). They consist of C_{25}, C_{26}, C_{27} and C_{28} sterols all of which have the 3,7,12-trihydroxycholane structure in common.

Recent investigations carried out in this laboratory indicated that C_{25} and C_{26} bile sterols in the bile of bull frog are derived from cholesterol in the organism (*4*). In the bile of toad there are pentahydroxycoprostane (*8*) which has C_{27} skeleton of cholesterol, and pentahydroxybufostane (*3*) which has an extra methyl group in addition to C_{27} skeleton of cholesterol. It seems therefore to be very important to know whether these bile sterols originate from cholesterol or not.

The present paper shows that radioactive pentahydroxycoprostane was isolated from the bile of toad after injection of cholesterol [4-C^{14}].

EXPERIMENTAL

Pentahydroxycoprostane was isolated from the bile of 100 toads. The bile of the animals was collected and extracted with 5 volumes of ethanol. The extract was evaporated to dryness, and hydrolyzed with 2.5 *N* sodium hydroxide for 8 hours at 160°C. Alkali-insoluble material containing neutral sterols was collected by filtration, dried, and subjected to reversed-phase column partition chromatography with phase system C (chloroform : isooctanol=15 : 15 as stationary phase and methanol : water=150 : 150 as moving phase), Hostalene being used as supporting material (*5*). Each 10 ml. portion of the eluate was collected by an automatic fraction collector and the concentration of bile sterols in each fraction was determined by measuring optical density at 320 mμ after treatment with sulfuric acid (Fig. 1) (*6*).

The effluents from 39.4 ml. to 58.0 ml : (the peak at 46.0 ml.) were collected, evaporated to dryness, and the residue was crystallized from ethyl acetate to prismatic crystals of m.p. 178°C.

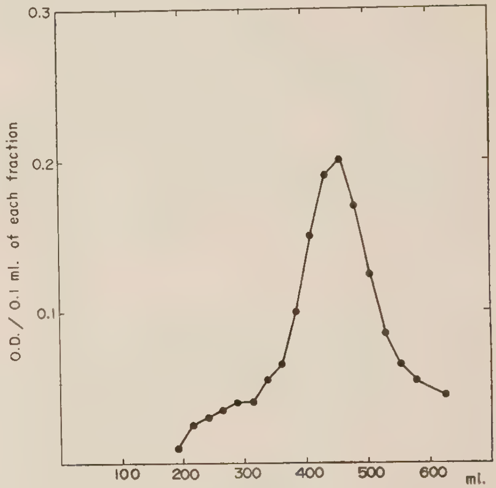

FIG. 1. Chromatography of bile sterols obtained from 100 toads.

Column : 45 g. of Hostalene, phase system ; C

Analysis Calcd. for $C_{28}H_{50}O_5$ C 72.06 H 10.80
Found : 71.60 H 10.77

Melting point of this sterol was not depressed when admixed with pentahydroxycoprostane which was isolated by Kazuno *et al.* (*8*). From these results this material was confirmed to be pentahydroxycoprostane (*8*).

Cholesterol [4-C^{14}] was purchased from the Radiochemical Centre, Amersham, England. Radioactive cholesterol (0.2 mc., 1.5×10^{8} c. p. m.) as an ethanolic suspension was injected intraperitoneally into 5 toads and the animals were kept together in a restraining cage for two weeks. The bladder bile of these animals was collected and extracted with 5 volumes of ethanol. The extract was evaporated to dryness, the residue was extracted with petroleum ether to remove unchanged cholesterol, and treated by the same method as mentioned above. Concentration of a neutral metabolite in each chromatographic effluent was determined by counting radioactivity by use of a gas flow counter.

RESULTS

Fig. 2 shows the chromatogram of bile sterols obtained from the bile of toads after injection of cholesterol [4-C^{14}].

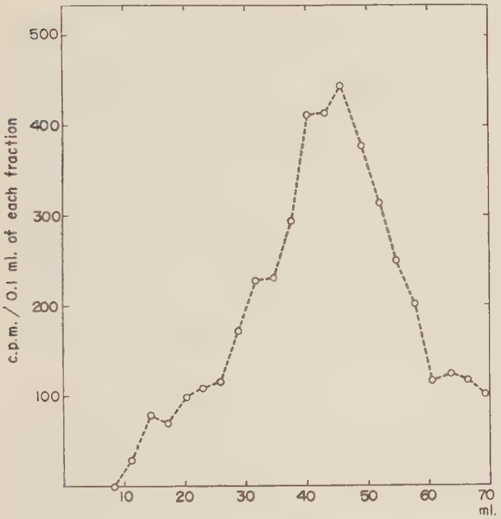

FIG. 2. Chromatography of the bile sterols of toads after injection of cholesterol [4-C^{14}].
Column: 4.5 g. of Hostalene, phase system: C

As shown in this graph, radioactivity was found in the effluents from 25 ml. to 60 ml. (the peak at 46.7 ml.). The total activity of this band was 1.1×10^5 c.p.m., corresponding to 1/1.35×10^3 of injected cholesterol [4-C^{14}]. The labeled material obtained from this band was identified with pentahydroxycoprostane by paper chromatography (70% acetic acid as stationary phase and ethylene chloride: heptane=60:40 as moving phase, (7)) and by subsequent actigraphy after dilution with 5 mg. of unlabeled pentahydroxycoprostane (Fig. 3).

As shown in Fig. 3 only one spot was found on the paper chromatogram which was in correspondence with the radioactivity on the actigram.

Further confirmation was obtained by cocrystallization to constant specific activity after dilution of the labeled material with 70 mg. of unlabeled pentahydroxycoprostane (Table I) and by subsequent rechromatography of the recrystallized material (Fig. 4).

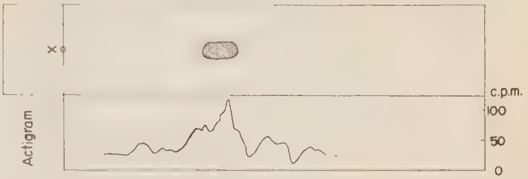

FIG. 3. Paper chromatography and actigraphy of the labeled material present in the effluents from 25 ml. to 60 ml. shown in Fig. 2.

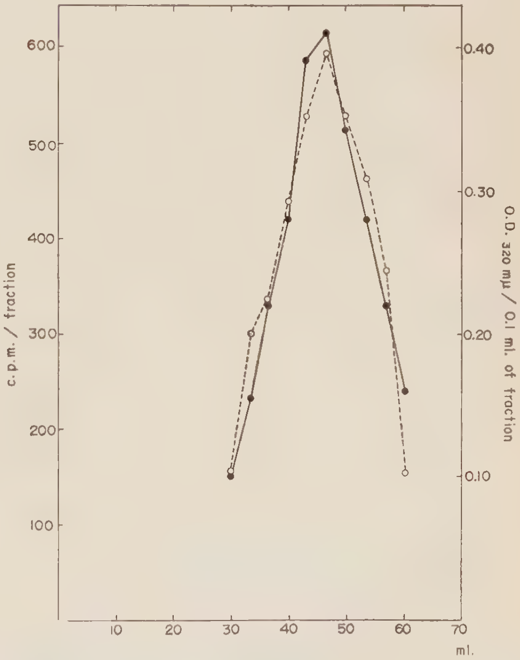

FIG. 4. Rechromatography of the labeled material in the effluents from 25 ml. to 60 ml. (Fig. 2) after its dilution with unlabeled pentahydroxycoprostane
Column: 4.5 g. of Hostalene, phase system: C; broken line, radioactivity.

A portion of the radioactivity in the original bile sterol fraction was retained in the stationary phase of the column (Fig. 2).

TABLE I

Successive Crystallization of 4-C^{14}-Pentahydroxycoprostane from Various Solvents

No.	Crystallizon solvent	Weight (mg.)	Total activity (c. p. m.)	Specific activity (c.p.m./mg.)
1	ethyl acetate	70.2	17,000	242
2	acetone	42.5	9,650	227
3	ethyl acetate	30.8	8,250	268
4	ethyl acetate	28.0	9,800	243

The column was eluted with ethanol and its residue was rerun with phase system F (chloroform: heptane=45:5 as stationary phase and methanol: water=165:135 as moving phase) (Fig. 5). As shown in the figure the radioactivity was found in 2 or 3 peaks. Isolation of 4-C^{14}-pentahydroxycoprostane from these peaks is now in progress.

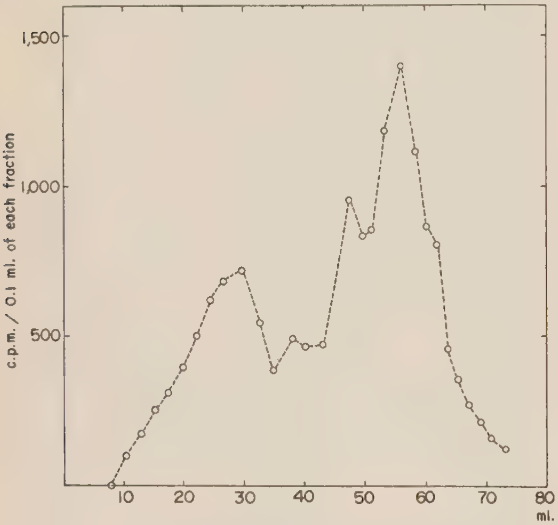

FIG. 5. Rechromatography of the residual material from the column shown in Fig. 2.
Column: 4.5 g. of Hostalene
Phase system: F

DISCUSSION

It was clearly demonstrated in the present work that the C_{27} bile sterol, such as pentahydroxycoprostane, is derived from cholesterol in the toad. The main two components of the bile sterols of toad are pentahydroxycoprostane and pentahydroxybufostane, and other sterols are only minor components.

According to our recent investigation, the structure of pentahydroxycoprostane proved to be 3α, 7α, 12α, 25ξ, 26-pentahydroxycoprostane (*8*). Therefore, the present result indicates an example of new type of catabolism of cholesterol which was not known so far.

SUMMARY

Cholesterol [4-C^{14}] was injected intraperitoneally into 5 toads and several radioactive bile sterols were isolated from their bile, one of them being identified with pentahydroxycoprostane.

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Studies on the Metabolism of Rat Ascites Tumor with Nitrogen Mustard Sensitive and Resistant Strains

V. P^{32}-Incorporation into Ribonucleic Acid *in Vitro*

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A wide variety of chemical agents such as the sulfur and nitrogen mustards, epoxides and ethyleneimines, possess a common ability to react *in vitro* with amino acids, proteins and nucleic acids (*1–3*). They have been shown, moreover, to elicit diverse biological effects, notably the production of mutations and chromosome aberrations, growth inhibition, and carcinogenic reactions (*4*). While the biochemical mechanisms underlying the effects of these alkylating agents are not known, biological activity as measured by the inhibition of tumor-growth or by the induction of chromosome abnormalities has been shown by *in vitro* studies to correlate with the reaction to nucleic acids. It has furthermore been suggested that the cellular sites of reaction may be ionized acid groups like either phosphate groups of nucleic acid (*5*) or alternatively, purine nucleotides (*6*, *7*).

The work described here concerns the investigation of the incubation media which permits the incorporation of P^{32} into RNA, as well as the change of the ratio of purines to pyrimidines in the ribonucleic acid of a nitrogen mustard sensitive strain of hepatoma after *in vitro* exposure to nitrogen mustard. The inhibition of P^{32}-incorporation *in vitro* into ribonucleotides of hepatoma cells by the addition of nitrogen mustard to the incubation medium was also investigated.

MATERIALS AND METHOD

The strains of rat ascites hepatoma cells used in the present work were originally obtained from the Sasaki Institute and were maintained by intraperitoneal injection in rats every 10 days. Yoshida Sarcoma cells were harvested 5 days after implantation. The ascites cells of hepatoma (strain AH 130 and AH 7974) were removed after 8–10 days' growth and freed from ascitic fluid and blood elements by centrifuging at 650×*g* for 5 minutes and by repeated washings with isotonic saline. Finally, the packed cells were diluted to an appropriated volume with calcium-free Krebs-Ringer-solution and added to the following incubation media.

Three kinds of incubation media which are shown in Table I were used to find out the best composition for the P^{32}-incorporation into ribonucleic acid (RNA). A total of 15 ml., 50 μc. of $Na_2HP^{32}O_4$ was added and such a cell suspension was incubated at 37°C for 1 hour under oxygen gas.

Table I

Composition of the Incubation Media to Compare the Incorporation

	Medium 1	Medium 2	Medium 3
Krebs-Ringer	5 vol.	6 vol.	3 vol.
5% Glucose	50 vol.	50 vol.	20 vol.
Ascitic fluid	—	10 vol.	—
Synthetic medium[1]	—	—	10 vol.

[1] 0.06 M KCl, 0.03 M $MgCl_2$, 0.001 M ATP-K, $1.3\times10^{-5}M$ Cytochrome c, 0.0067 M α-Ketoglutarate

To test the inhibition of nitrogen mustard on the P^{32}-incorporation, another kind of incubation medium was adopted: ascitic fluid containing hepatoma

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cells, 5 ml., Krebs-Ringer-phosphate (pH 7.4), 1 ml., 0.9% NaCl, 2.5 ml., 25 μc. $Na_2HP^{32}O_4$, total volume: 8.5 ml.

In Medium 2 of Table I, the ascitic fluid was substituted by sera or saline which was injected into the abdominal cavity of a normal rat and was expired after 20 minutes. Liver-press-juice which was used once as a substitute for ascitic fluid, was made by the method of Kaltenbach (*8*) and centrifuged off the isolated cells. Dialyzable substances of ascitic fluid were obtained by the dialysis of 20 ml. of ascitic fluid in a cellophane bag against isotonic sucrose solution for 24 hours at 4°C. After the incubation, the hepatoma cells were collected by low speed centrifugation and nucleic acids were extracted by Kirby's (*9*) phenol method. In the case of the experiments for the comparison of incubation media, inorganic phosphate contaminated in the RNA fraction was removed by means of a paperelectrophoresis (*10*) and the amount of RNA was determined by Bial's orcinol method. The phosphorus in RNA was calculated as 8 per cent of the total weight of RNA.

In the case of the experiments for the determination of inhibitory effects of nitrogen mustard, RNA was decomposed by alkali treatment. Then the mixture of mononucleotides was fractionated by means of Dowex-1 column with continuous elution of 1 to 4 *N* formic acid. Inorganic phosphate which has been contaminated into RNA fraction was completely removed from mononucleotides by this procedure (*10*). The amount of mononucleotides was measured spectrophotometrically using a "Hitachi" Beckman Type electrophotometer and the amount of phosphorus in mononucleotides was calculated. The radio-activity was measured by G-M counter and from the ratio of the specific activities of mononucleotides and those of the incubation medium, the relative specific activity was calculated.

RESULTS

The results obtained by altering the composition of the medium in 1-hour incubations of ascites cell suspensions with P^{32} are shown in Table II.

It is evident that in Krebs-Ringer-glucose medium supplemented with ascitic fluid there is a greater incorporation of P^{32} into RNA than in the pure synthetic Krebs-Ringer-glucose medium. The addition of ascitic fluid caused an appreciable increase in the relative specific activity of RNA.

Table II

Comparison of Relative Specific Activity[1)] *of RNA after 1-Hour Incubation of Ascites Cells in the Two Kinds of the Incubation Media*

Tumor	Krebs-Ringer-glucose	Krebs-Ringer-glucose +Ascitic fluid
AH 130	2	60
	2	60
	2	50
AH 7974	—	30
	2	30
	—	20
	1	10
Yoshida Sarcoma	5	50
	—	50
	1	20

1) Relative specific activity= $\frac{\text{sp. ac. of RNA-P}}{\text{sp.ac. of Medium-P}} \times 10^5$

Since Warburg *et al.* (*10*) and Burk (*11*) preferred to carry out ascites cell incubations in pure ascites serum, this procedure has been adopted by Harrington and Lavik (*12*), and Harbers and Heidelberger (*13*). Attempts were made to develope a medium for incubation of ascites cell suspensions without using ascites fluid which varies its features case by case.

Table III shows the effects of the various ascitic fluid, sera or liver-press-juice on the relative specific activity of RNA-P^{32} in Yoshida Sarcoma cells. The experimental conditions were same as in Table II.

The addition of biological fluid from normal rats caused a slight increase in the relative specific activity of RNA-P^{32}. However, when biological fluid issued from tumor bearing rats or regenerating rat liver was added to the incubation media, an appreciable increase in relative specific activity of RNA-P^{32} was observed. The substance or substances which stimulate the incorporation of P^{32} into RNA might be a low-molecular compound(s) since the dialyzable substances from ascites fluid were more effective than the non-dialyzable substances.

When Medium 3 which contains no ascitic

TABLE III

Effects of Various Ascitic Fluids, Sera and Liver-Press-Juice on the Relative Specific Activity of RNA-P^{32} in Yoshida Sarcoma Cells

Biological fluid added	Relative specific activity1) of RNA-P^{32}
Ascites fluid from normal rats	7
from tumor bearing rats	30
Ascites fluid, non-dialyzable substances	5
dialyzable substances	20
Sera from normal rats	10
from tumor bearing rats	30
Liver-press-juice of normal rat liver	10
of regenerating ral liver	30

1) Same to that of Table II

fluid was used, the relative specific activity of RNA-P^{32} was about 10 times higher than in Medium 1 (Krebs-Ringer-phosphate-glucose). Consequently, it was decided to follow the substance which exists in Medium 3 and stimulates the incorporation of P^{32} into RNA, although there might be many natural stimulating substances in the biological fluid.

Table IV shows the stimulating effects of adenine deviratives for the incorporation of P^{32} into RNA of Yoshida Sarcoma cells.

It may be seen from the results shown in Table IV that the main stimulating effect of Medium 3 is due to the existence of ATP. This stimulating effect was also observed more or less when the medium containing adenine derivatives like adenosine, adenylic acid and adenosine diphosphate was used but guanine did not show any enhancing effect for the incorporation of P^{32} into RNA.

Although the use of ATP could replace a biological fluid like ascites or sera, excessive use of ATP might interfere with the effect of nitrogen mustard since the effect of nitrogen mustard on the protein synthesis of cancer cells was slightly inhibited by the addition of ATP into the incubation medium. Moreover, as cited by Creaser, de Leon and Scholefield (*14*), the incorporation of P^{32} into nucleic acids probably occurred mainly by exchange when the cells were incubated in salt alone, whereas much greater *de novo* synthesis occurred when ascitic fluid was present. The addition of ATP to the medium did increase the incorporation of P^{32} into RNA but still there is a lack of evidence that ATP is equivalent to ascitic fluid for the maintaining of *de novo* synthesis of RNA.

TABLE IV

The Stimulating Effects of Adenine Derivatives for the Incorporation of P^{32} into RNA of Yoshida Sarcoma Cells

Composition of media Basal medium (Medium 1)	Relative specific activity1)
B. M.+α-Ketoglutarate, Cytochrome c & ATP	10
B. M.+Cytochrome c, ATP	10
B. M.+α-Ketoglutarate, ATP	10
B. M.+α-Ketoglutarate, Cytochrome c	3
B. M.+ATP	28
B. M.+ATP	33
B. M.+ATP	38
B. M.+ADP	10
B. M.+AMP	12
B. M.+Adenosine	14
B. M.+Adenine	13
B. M.+Guanine	2

1) Same to that of Table II

Consequently, Medium 2 was used as the incubation medium for the experiments in which the effect of nitrogen mustard was tested.

The data given in Table V show the addition of $10^{-3} M$ nitrogen mustard or $10^{-3} M$ thio-TEPA* into the incubation medium of rat ascites hepatoma cells. The cells used for the test of the inhibitory effect of the alkylating agents and those used for the control were issued from the same animal. Thus they were able to be compared with each other although the metabolic activities of hepatoma cells vary usually from animal to animal.

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* Triethylenimino thiophosphoramide, Sumitomo Chemicals & Co.

TABLE V

The Effect of $10^{-3}M$ Nitrogen Mustard and Thio-TEPA on the Incorporation of P^{32} into Monoribonucleotides of Rat-Ascites Cells after 1-Hour Incubation in Medium Containing Ascitic Fluid

Ascitic fluid Rat-ascites-hepatoma	Relative specific activity[1]			
	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid
AH 130	4.2	4.5	5.2	2.0
AH 130 with $10^{-3}M$ nitrogen mustard	2.7	2.5	2.9	9.0
AH 130	13.0	13.0	15.0	25.0
AH 130 with $10^{-3}M$ Thio-TEPA	7.8	4.8	7.2	16.0
AH 7974	4.8	5.4	5.8	20.0
AH 7974 with $10^{-3}M$ nitrogen mustard	3.6	5.1	4.1	11.0
AH 7974	4.9	4.5	7.3	11.0
AH 7974 with $10^{-3}M$ Thio-TEPA	2.5	3.1	3.2	8.0

1) Relative specific activity= $\frac{\text{sp. ac. of mononucleotide-P}}{\text{sp. ac. of medium-P}} \times 10^5$

In every trial of the experiments, the addition of the alkylating agents to the medium lowered the rate of incorporation of P^{32} into mononucleotides. However there was a slight difference between the incorporation of radioactivity into mononucleotides of nitrogen mustard sensitive (AH 130) and resistant (AH 7974) strains of hepatoma: the mean inhibition rate was around 45 per cent in the sensitive strain whereas it was around 33 per cent in the resistant strain.

The molar ratio of mononucleotides of the sensitive hepatoma cells also changed after the incubation with $10^{-3}M$ alkylating agents. Table VI shows the molar ratio of monoribonucleotides of hepatoma cells after 1-hour incubation with or without addition of alkylating agents. In every case, the amounts of adenylic acid was assumed as 10 and the amount of other mononucleotides was calculated.

It can be seen that the hepatoma RNA preparations of both strains were rich in guanylic acid and cytidylic acid, and poor in adenylic acid and uridylic acid. The ratio of purines to pyrimidines was generally a little bit higher from unity.

The ratio of guanylic acid to adenylic acid was around 2, which may suggest that the pattern of the mononucleotides ratio of hepatoma would be similar to that of liver except for high purines to pyrimidines ratio which were around 1.15—1.30. According to the study of Volkin and Carter (15), the ratio of purines to pyrimidines in normal rat liver is around 1.04 and in regenerating rat liver, 1.05.

The most interesting observation is the fact that the high ratio of purines to pyrimidines decreased to the level of unity in the nitrogen mustard sensitive strain (AH 130) after the treatment of $10^{-3}M$ of alkylating agents whereas the ratio was still higher than unity in the resistant strain (AH 7974) even after the treatment of alkylating agents. This is apparently due to the relative increa-

TABLE VI

The Ratio of Mononucleotides after 1-Hour Incubation of Rat-Ascites-Hepatoma with or without the Addition of Alkylating Agents

Rat-ascites-hepatoma	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	Purines / Pyrimidines
AH 130	10	20	12	11	1.30
〃	10	20	13	8	1.42
〃	10	20	16	10	1.15
AH 130 with $10^{-3}M$ nitrogen mustard	10	22.6	23.8	11.2	0.93
AH 130 with $10^{-3}M$ Thio-TEPA	10	23.7	18.8	15.0	1.00
AH 7974	10	20	14	20	1.30
〃	10	20	16	10	1.15
AH 7974 with $10^{-3}M$ nitrogen mustard	10	20	15	11	1.15
AH 7974 with $10^{-3}M$ Thio-TEPA	10	11.7	10.8	5.8	1.30

se of pyrimidine nucleotides after the treatment with alkylating agents in the case of nitrogen mustard sensitive strain.

DISCUSSION

The results reported above have been developed in three main phases: (a) an investigation of the incubation media for the *in vitro* incorporation of P^{32} into the RNA of tumor cells, (b) determination of the inhibitory effects of nitrogen mustard on the P^{32} uptake of ribonucleotides, and (c) the change of the mononucleotides ratio by the addition of alkylating agents.

According to the recent study of Sibatani *et al.* (*16*), the RNA isolated from the aqueous layer of Kirby's phenol preparation shows a relatively lower metabolic rate similar to that of DNA. In fact, in our case also, the relative specific activity of tumor RNA was generally lower than those of RNA extracted by Schmidt-Thannhauser's method.

However as seen in Table II and III, the addition of serum or ascitic fluid preferably those taken from tumor-bearing-animals increased ten times or sometimes more the relative specific activity.

Katsuta *et al.* (*17*, *18*) using a replicate tissue culture and fluid-suspension culture demonstrated the requirement of serum protein for the propagation of rat ascites hepatoma cells (AH 130 and Yoshida Sarcoma cells). However, from the fact as they succeed to substitute polyvinylpyrrolidone or dextran for serum protein, they suggested that most parts of serum proteins are essential as a physicochemical component rather than as nutritives to the propagation of these cells.

The propagation of tissue culture cells and the labelling with P^{32} of RNA are essentially different system each other but we could assume that the physico-chemical components would be necessary to maintain the proper metabolism of ascites cells. Thus, as Creaser, de Leon and Scholefield (*14*) demonstrated, the presence of ascitic fluid might not affect the rate of P^{32} incorporation into nucleic acids but might affect the total amount incorporated by prolonging the period during which incorporation takes place at the initial high rate.

Evidence is lacking for the identification of adenine derivatives as the principal effective compnents of ascitic fluid for the P^{32}-incorporation into nucleic acids. However, from the view point of the increasing capacity of the relative specific activity of RNA, adenine derivatives were able to be substituted for ascitic fluid. As cited before, this increasing uptake of P^{32} might occur predominantly by an increase of exchange but not by the stimulation of *de novo* synthesis, since not only ATP but also adenine, adenosine, (after phosphorylation), adenilic acid or ADP, might be the supplyer of exchangeable P^{32}.

The relative specific activities of the nucleotides of AH 130 and AH 7974 did not show marked differences between the two strains. In a few cases, the high specific activity was observed in uridylic acid. Yamana and Sibatani (*19*) or Hadjiolov and Ilkov (*20*) showed similar deta with liver, lymphatic cells and Ehrlich ascites tumor cells.

The concentration of nitrogen mustard used in this experiment ($10^{-3}\,M$) was too high from the physiological view point, since the minimum effective dose *in vivo* is around 10^{-7} *M*. However, in this low concentration of nitrogen mustard, inhibitory effect of the incorporation of P^{32} into RNA was scarecely detected.

On the other hand, the energy-yielding system of hepatoma cells is largely affected by the addition of $10^{-3}\,M$ nitrogen mustard to the incubation medium (*20*, *21*). Thus, the inhibition of the incorporation of P^{32} into RNA might be at least partially due to the inhibition of the energy-yielding system. Consequently, the inhibitory effect of nitrogen mustard on the RNA metabolism would not be conclusive. However, the change of the ratio of purines to pyrimidines in AH 130 cells after the treatment with nitrogen mustard is likely due to the direct effect of this compound, since this effect was not obserbed in the case of the nitrogen mustard resistant strain.

Lawley and Wallick (*22*) reported the susceptibility of guanine in DNA to the addition of nitrogen mustard. If the guanine of RNA is also susceptible to nitrogen mustard, it might give some hints to explain the increased amount of guanylic acid and cytidylic acid of AH 130 RNA after the treatment of alkylating agents (Table VI), since cytidylic acid might be the pair nucleotide with guanylic acid.

The ratio (G+C): (A+U) is 2.00 in normal rat livers, 1.93 in regenerating rat livers, 1.71 in AH 130, 1.47 in AH 7974 and 1.59 in Ehrlich ascites tumor (*20*). When alkylating agents were added, the ratio became 1.94 in AH 130, the value closed to that of the regenerating liver whereas in AH 7974 the ratio remained at the lower level of 1.55. These facts may suggest that nitrogen mustard does not penetrate sufficiently into the cells of resistant strain.

SUMMARY

1. The composition of the incubation medium for the P^{32}-incorporation into ribonucleotides of rat ascites tumor was studied. The relative specific activity increased about ten times when sera or ascites fluid was added to the incubation medium. The addition of adenine derivatives apparantly increased the incorporation but evidence was lacking as this was merely based on the increasing exchange reaction.

2. The addition of nitrogen mustard to the incubation medium inhibited the P^{32}-incorporation into four ribonucleotides. The change of nucleotides ratio in susceptible cells was obserbed. The ratio of purines to pyrimidines or that of (G+C):(A+U) was modified by the addition of nitrogen mustard in the incubation medium of the sensitive strain.

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Studies on the Metabolism of Rat Ascites Tumor with Nitrogen Mustard Sensitive and Resistant Strains

VI. Inhibition of the *in Vitro* Incorporation of C^{14}-Orotic Acid into Nucleic Acids by Nitrogen Mustard

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Many types of experimental investigations have implicated nucleic acid metabolism as a possible site for the action of alkylating agents on neoplastic tissue (*1–6*). The fact that alkylating agents inhibit the biosynthesis of deoxyribonucleic acid (DNA) but do not affect ribonucleic acid (RNA) metabolism suggests that the anticancer specificity exhibited by these agents may result from quantitative or qualitative differences between normal and neoplastic cells in regard to the biosynthesis of DNA. However, it was found that animals bearing a strain of ascites hepatoma, AH 7974, are resistant to nitrogen mustard, whereas this agent is most effective on another strain of hepatoma, AH 130 (*7*).

It was expected, therefore, that the biosynthesis of DNA in AH 7974 cells would not be affected by the addition of nitrogen mustard, whereas this agent would inhibit the biosynthesis of DNA in AH 130 cells. However, it was not clear whether this resistant character of AH 7974 cells depends on the permeability of nitrogen mustard into the cells or on the existence of an alternative pathway of DNA synthesis.

With this concept in view, the following studies on the *in vitro* uptake of a labeled precursor of nucleic acids by hepatoma cells, homogenates and isolated nuclei were undertaken. The specific objective of this program was to search for the existence of a barrier system for nitrogen mustard which might be expected preferentially on the surface of AH 7974 cells or on their nuclear membrane. It is known that orotic acid incorporated into the nucleic acids of cancer cells (*8*). However, little data have been available for the utilization of the precursor by isolated cancer nuclei (*9*).

MATERIALS AND METHODS

Rat ascites hepatoma cells (AH 130 and AH 7974) were taken from female rats 5–8 days after transplantation. In the case in which the cell-suspension was used for the incorporation experiments, the cells were not cooled in an ice bath but were maintained at room temperature until the beginning of the incubation. The combined ascites from serveral rats were mixed and then centrifuged for 5 minutes at 1,000×*g*. The supernatant ascites serum was used for the dilution of the incubation medium. One ml. of the sedimented packed cells was suspended in 1.0 ml. of Robinson's medium containing 0.05 ml. of chick embryo extract (lyophilized extract EE 100 from Difco Laboratories in 2 ml. of water), then the suspension was added 1 ml. of ascites serum (*10*). The Robinson's medium without bicarbonate contained the labeled precursor (1.28 μmole 92,500 c.p.m.) per vessel of orotic acid-2-C^{14}) (*11*), glucose (3 mg. per vessel) and other compounds like nitrogen mustard as necessary. The final pH was adjusted to pH 7.4.

In the case of the homogenate of the hepatoma cells, cooled hepatoma cells were homogenized with 0.25 *M* sucrose and made 30% homogenate. One ml. of the 30% homogenate was combined with 1 ml. of Robinson's medium and 1 ml. of ascites serum.

To make the nuclear fraction, sedimented cells were washed for short time with cold water and then suspended in 0.25 *M* sucrose containing 0.0018 *M* $CaCl_2$. This suspension was added thouroughly on the layer of 0.35 *M* sucrose. After careful centrifugation for 10 minutes at 1,500×*g* the sedimented nuclei were homogenated in 0.25 *M* sucrose with a tight Teflon pestle homogenizer and filtered through a cotton flannel, then resuspended on the layer of 0.35 *M* sucrose.

The centrifugation for 10 minutes at 1,500×*g* was carried out again and the collected nuclei were resuspended in 0.25 *M* sucrose. One ml. of the suspension was combined with 1 ml. of Robinson's medium and 1 ml. of ascites serum.

The incubations were carried out at 37°C for 3 hours with constant shaking under O_2 gas. The reaction was stopped by addition of equal volume of 8% perchloric acid (PCA) containing C^{12}-orotic acid. The acid-soluble fraction was removed by successive washings with cold 4% PCA, 2% PCA, 1% PCA and 1% PCA with ethanol (1 : 1 v/v). The lipidic fraction was then removed successively with ethanol, ethanol-chloroform (3 : 1 v/v), acetone, alcohol-ether (3 : 1 v/v) and ether. The extraction of nucleic acids was carried out in equal volume of 0.9% NaCl and 5 g./dl. "Duponol" (5 g. Na salt of lauryl sulfate in 100 ml. of 45% ethanol) at 100°C for 10 minutes, then more NaCl was added to the suspension to make the final concentration of NaCl to 1 molar concentration. One sixth volume of chloroform was added with vigorous shaking for 10 seconds. After centrifugation, the supernatant layer was pipetted and an equal volume of acetone and two volumes of alcohol were added to the extracted solution to make sediment. After centrifugation, the sediments were dissolved again with 10 volumes of cold water. This aqueous solution was acidified with 1/10 volume of 8% PCA and allowed to stand overnight in the cold. The precipitated nucleic acids were then digested with 2 ml. of *N* NaOH for 16 hours at 37°C. DNA was precipitated by the acidification with 0.4 ml. 6 *N* HCl and 2 ml. of 8% PCA. After centrifugation, the sediment of DNA was washed with ethanol. The mixture of mono-ribonucleotides was precipitated by the addition of 4 volumes of ethanol. The quantitative determinations of nucleic acids were carried out by means of an "Hitachi" Beckman type spectrophotometer at 260 mμ. The calculations were done for RNA at pH 2, $\varepsilon(P)=9{,}800$ and for DNA at pH 12, $\varepsilon(P)=8{,}270$. Aliquots of each DNA and RNA were plated on aluminum planchets and the radioactivity was determined on a proportional gas counter ("Ten" type EA-102, Kobe Kogyo) at infinite thinness.

RESULTS

The results of the preliminary experiments obtained by altering the composition of the medium or the incubation time are omitted here. It was, however, shown that the optimal condition so far tested for incorporation of the labeled precursor into isolated nuclei was obtained under the conditions described in the section of method; *e.g.* 3-hours incubation under oxygen gas in the incubation medium containing glucose, ascitic fluid, and chick-embryo-extract, *etc.*.

Practically no radioactivity was observed in the final samples when the reaction mixture was not incubated (O-time experiment) if the aliquots were washed with the carrier containing PCA. The actual count of the final aliquots was usually around 30–100 c.p.m. (without containing background counts).

The data presented in Table I shows the results obtained with hepatoma cell suspension. The relative specific activity of DNA in AH 130 was about one third of that of RNA whereas in AH 7974 both DNA and RNA showed about the same relative specific activity.

TABLE I

Incorporation of C^{14}-Orotic Acid into Nucleic Acids of Hepatoma Cells

Rat ascites hepatoma	Concentration of nitrogen mustard	RNA		DNA	
		c.p.m./ μmoleP	%	c.p.m./ μmoleP	%
	none	15.1	100	5.5	100
AH 130	10^{-5} *M*	14.4	96	6.6	119
	10^{-4} *M*	4.0	27	3.2	58
	none	12.0	100	11.8	100
AH 7974	10^{-5} *M*	27.2	227	29.3	248
	10^{-4} *M*	9.4	78	10.2	87

The addition of nitrogen mustard to the incubation medium at the concentration of 10^{-5} *M* was more stimulative than inhibitory, especially in the case of AH 7974. The effect of more concentrated nitrogen mustard (10^{-4} *M*) was remarkably inhibitory in the case of sensitive AH 130 but slightly inhibitory in the case of resistant AH 7974. The direct action of nitrogen mustard to the biosynthetic system of nucleic acids was demonstrated in Table II using homogenates of hepatoma. The remarkable inhibitory action by 10^{-4} *M* nitrogen mustard for both RNA and DNA of resistant AH 7974 was impressive in this experiment. This fact suggests that the nature of drug resistance might be consisted in the permeability of cell membrane.

TABLE II

Incorporation of C^{14}-Orotic Acid into Nucleic Acids of Hepatoma Homogenate

Rat ascites hepatoma	Concentration of nitrogen mustard	RNA		DNA	
		c.p.m./ μmoleP	%	c.p.m./ μmoleP	%
AH 130	none	9.4	100	4.1	100
	$10^{-5}\,M$	10.3	109	7.0	171
	$10^{-4}\,M$	5.5	58	1.8	45
AH 7974	none	220.0	100	36.9	100
	$10^{-5}\,M$	—	—	—	-
	$10^{-4}\,M$	39.6	18	2.0	5

If we assume that the lower concentration of nitrogen mustard stimulates nucleic acid metabolism and the higher concentration of the drug suppress the metabolism, the results obtained in the Tables I and II would be easily explicable. This might be interpreted as the existence of some kind of affinity to nitrogen mustard in the superficial structure of sensitive strain (*12*).

The data obtained with isolated nuclei are shown in Table III. The incorporation of the labeled precursor into both DNA and RNA of the two strains was remarkably inhibited by the addition of $10^{-5}\,M$ nitrogen mustard. DNA was affected rather deeply as compared with nuclear RNA. The fact that there was no stimulatory effect in the incorporation is interpreted as follows.

TABLE III

Incorporation of C^{14}-Orotic Acid into Nucleic Acids of Hepatoma Nuclei

Rat ascites hepatoma	Concentration of nitrogen mustard	RNA		DNA	
		c.p.m./ μmoleP	%	c.p.m./ μmoleP	%
AH 130	none	166.4	100	16.9	100
	$10^{-5}\,M$	88.1	53	6.9	41
	$10^{-4}\,M$	74.5	45	3.5	21
AH 7974	none	299.0	100	103.5	100
	$10^{-5}\,M$	263.0	88	27.2	26
	$10^{-4}\,M$	—	—	—	—

Since the naked nuclei has no barrier system against the penetration of nitrogen mustard, the more concentrated drug reached directly to the enzyme system for the biosynthesis of both DNA and RNA. Therefore no stimulatory phase occurred. Another interpretation will be discussed in the following section.

DISCUSSION

There are several methods of isolating nuclei from various kinds of tissue. Busch, Starbuck and Davis (*13*) obtained the isolated nuclei of the purity of 99% from Walker's carcinosarcoma 256 having used homogenizers of appropriate pestle clearance. However, the use of $2.0\,M$ sucrose might not be fitted for incorporation experiments *in vitro* with isolated nuclei.

Roff and Aub (*14*) utilized Allfrey and Mirsky's method (*15*) to isolate nuclei and obtained good results for the uptake of amino acids into protein. Recently, Rees and Rowland (*9*) adoptep a method for the isolation of rat-liver-nuclei in $0.25\,M$ sucrose with the simultaneous use of nylon bolting cloth (60 and 110 mesh/in.) for filtration. By this method, the isolated nuclei incorporated P^{32}, 8-C^{14}-adenine, 6-C^{14}-orotic acid and 2-C^{14}-glycine into their nucleic acids. In this case, the use of $0.34\,M$ sucrose did not disturb those incorporations.

Our method for the isolation of cancer-cell-nuclei is originally based on Allfrey, Mirsky and Osawa's method (*15*). A microscopic examination was made and very few erythrocytes were observed but no cellular debris was contaminated.

The incorporation rate of C^{14}-orotic acid into DNA and RNA was different from experiment to experiment even when the experiment was done at the same day after the inoculation of cancer. However the relative effect of nitrogen mustard as expressed in per cents was relatively constant even when the absolute incorporation varies in a wide range.

Among the data presented in Tables I—

III, the stimulatory effect of nitrogen mustard seems rather strange. However, the enhancing effect of nitrogen mustard on the P^{32}-incorporation into RNA was already reported by Heidelberger and Keller (*6*) in 1955. Recently, Henderson and Junga (*16*) observed a marked stimulation of isotope incorporation into RNA and DNA by the administration of 4-aminopyrazolo (3, 4-d) pyrimidine or 1-methyl-4-aminopyrazolo (3, 4-d) pyrimidine.

The irradiation which gives generally a similar effect as nitrogen mustard, produces also a stimulation of total RNA biosynthesis (*17*). Vermund *et al.* (*18*) investigated the effects of irradiation on the utilization of P^{32} by different cell fractions of a mouse mammary carcinoma and observed some inhibition of the P^{32}-uptake into nuclear RNA but they noticed simultaneously a stimulatory effect on the P^{32} incorporation into the soluble RNA. However, Thompson *et al.* (*19*) obtained opposite results in studies on the effects of irradiation on regenerating liver. Beltz *et al.* (*20*) reported a complete suppression of the incorporation of orotic acid into the DNA of regenerating liver of rats irradiated with 1,500 r., whereas the specific activity of the cytoplasmic RNA was unaffected.

Moreover, Harbers and Heidelberger (*10*) postulated that one type of nuclear RNA might be inhibited and the other type stimulated in its metabolism as a consequence of irradiation, which would result in the lack of effect of irradiation on RNA biosynthesis that has been most commonly reported. Furthermore, Lawley (*21*) demonstrated that deoxyguanylic acid is most susceptible to the alkylation of the nitrogen mustard.

Our results obtained with isolated nuclei showed that the incorporation of the labeled precursor into DNA was more susceptible to the addition of nitrogen mustard to the medium than that into RNA and that nuclear RNA was also affected by $10^{-5}\,M$ nitrogen mustard which gave usually a stimulation in the case of homogenates or cell RNA.

Emphasis should be laid on the fact that in the *in vivo* experiment, nitrogen mustard or its derivatives are as effective as an anticancer agent at the low concentration as $10^{-7}\,M$. This low concentration of nitrogen mustard would follow a stimulation of nucleic acid metabolism rather than an inhibition to this system. If this is true, some kind of the stimulation in the nucleic acid metabolism would result an anticancerous action in experimental chemotherapy.

Lack of substantial evidence for the existence of a barrier system on the surface of resistant AH 7974 cells led us to hesitate in demonstrating our view for the mechanism of drug resistance. In this connection, the experiment of Bieber, Dietrich, Elion and Hitchings (*22*) is interesting: during the course of the study on the incorporation of 6-mercaptopurine-S^{35} into DNA of sensitive and nonsensitive transplantable mouse tumors, they found no correlation between the concentration of 6-mercaptopurine incorporated and the degree of inhibition of the tumor. Furthermore, a mercaptopurine-resistant subline of adenocarcinoma 755 incorporated more of the analogue than did the sensitive line. Thus we are not able to say that resistant AH 7974 cells show some kind of resistance against the penetration of nitrogen mustard, until the direct evidence of the existence of a barrier system is obtained.

SUMMARY

1. The addition of $10^{-5}\,M$ of nitrogen mustard to the incubation media of rat ascites hepatoma cells or homogenates was accompanied by an increase in the uptake of C^{14}-orotic acid into RNA and DNA. But in the incubation of the isolated nuclei, the incorporation was inhibited by the same concentration of nitrogen mustard.

2. Increase in the concentration of nitrogen mustard ($10^{-4}\,M$) in the incubation media led to an inhibition of the incorporation. The extent of the inhibition was much less in the case of the nitrogen mustard resistant cells (AH 7974) than in sensitive cells (AH 130). However, this difference was not observed when the cells were once homogenated.

3. The resistant cells apparently have some barrier system on the surface of the cells for the penetration of nitrogen mustard into the inside of the cells. However direct evidence was lacking for the existence of this barrier system at the present stage of investigation.

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Inorganic Polyphosphate in Spinach Leaves

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Metaphosphate and inorganic polyphosphate are known to be widely distributed among lower plants such as moulds, bacteria and algae. Their occurrence in higher plants (ferns and seed plants), however, has not so far been detected, with the exception that Hardin (1892) reported the presence of metaphosphate in cotton seeds (see, *e.g.*, Schmidt (*1*), and Keck and Stich (*2*)).

Until recently, the biochemical studies of inorganic polyphosphate have been performed mainly with yeast and bacteria. On the basis of these studies, it is now generally agreed that polyphosphate plays an important role as energy reservoir in the microorganisms. During the last several years, evidence has been accumlating to indicate that, in algal cells, polyphosphate is synthesized, by transphosphorylation reactions, from phosphorylated intermediates resulting from the photosynthetic process, and plays an active role in the photo-induced intracellular phosphate transfer. It was found by Stich (*3*) that, when *Acetabularia* cells are illuminated, a considerable uptake of labeled phosphorus into polyphosphate takes place, whereas there occurs no noticeable incorporation of P^{32} in the dark. The extensive study by Wintermans (*4*) showed that, in *Chlorella* cells, the incorporation of phosphate into polyphosphate is most pronounced in the light and in the absence of CO_2. Our study with *Chlorella* has also indicated that, under photosynthesizing conditions, the phosphorus used in the synthesis of deoxyribonucleic acid (DNA) and phosphoprotein is primarily derived from polyphosphate (*5–6*). These results raise the question as to whether the presence and function of polyphosphate are limited to lower plants. To answer this question, spinach leaves were tested for their content of inorganic polyphosphate. The positive results obtained are briefly described in the present paper.

MATERIAL AND METHODS

The material used was the leaves of *Spinacea oleracea* L. purchased in the market during the period between mid-January and mid-April. The leaf-blades were ground in a Waring blender for 2 minutes with one to two times their weight of 0.01 *M* tris buffer (pH 8.2). The homogenate was filtered through two layers of gauze, and the coarse debris discarded.

The filtered homogenate was centrifuged at 25,000 ×*g* for 30 minutes; the "supernatant" showed only a faint yellowish-brown colour, indicating that chlorophyll-containing substances went almost exclusively into the "precipitate". Immediately afterwards, the phosphorous compounds in both the "supernatant" and "precipitate" were fractionated, according to the method of Schmidt and Thannhauser (*7*) which had been modified as follows (*6*).

The test materials were treated successively with: (I) trichloroacetic acid (TCA) (final concentration, 8%) three times, for 30 minutes each, (II) 75% and 95% ethanol, (III) hot ethanol-ether (3:1) (6 times), and (IV) dilute KOH of pH 9 (twice, for 30 minutes and 1 hour, respectively). The residue thus obtained was incubated with 2 *N* KOH at 37°C for 20 hours, and (V) the supernatant was neutralized with $HClO_4$. The amounts of orthophosphate, labile phosphate and total phosphorus were determined with the supernatants obtained by procedures IV and V. The labile phosphate was also determined with the precipitate obtained by procedure V ("$KClO_4$-fraction").

Determination of labile phosphates (Δ15P or Δ30P) were performed by measuring the quantity of orthophosphate liberated on boiling the samples for 15 (or 30) minutes in the presence of *N* H_2SO_4. Orthophosphate was determined according to the method of Berenblum and Chain (*8*) as modified by Yanagita (*9*) or according to the method of

Lohmann (*10*) as modified by Moriwaki (*11*).

In another series of experiment, the homogenate of leaves was treated with TCA immediately after filtration through gauze, and from the precipitate obtained, the ribonucleic acid- (RNA-) and DNA-fractions were seperated successively according to the method of Ogur and Rosen (*12*). Lipid was first removed from the precipitate by treatment with ethanol and ethanol-ether as described above; RNA was then extracted by incubating the material for 46 hours with 10% perchloric acid (PCA), and the resulting residue was extracted twice (20 minutes each) with 5% PCA at 70°C to obtain the DNA-fraction. The RNA- and DNA-fractions were adjusted to pH 4.0 with the addition of acetate buffer (pH 4.0). After adding a small quantity of commercial pyrophosphate (as co-precipitant for polyphosphate possibly existing in the samples), the mixture was shaken with $Ba(NO_3)_2$ and left overnight. The precipitate formed was collected by centrifugation, washed once with distilled water, and dissolved in water by mechanical shaking (for 4 hours) together with Amberlite IR-120 (H^+-form) (*13*). The ion-exchange resin as well as a small amount of turbid material which remained insoluble were removed by successive centrifugation and filtration. The clear solution thus obtained contained—as revealed by the observation of ultraviolet absorption—only a negligible amount of nucleic acid components but contained a sizable amount of polyphosphate, as will be shown later by the data of metachromatic reaction and of paperchromatography. Unless otherwise stated, the operations described above were always performed in the cold (lower than 5°C).

RESULTS

Labile Phosphorus Compounds in Acid-Insoluble Fractions—As will be seen from the results presented in Table I, the labile phosphorus compounds are contained in the supernatants obtained by Procedure IV ("pH 9-extract") and in the supernatants obtained by procedure V ("2 *N* KOH-extract") of both "supernatant" and "precipitate". Data which are not shown here indicate that only a negligible amount of labile phosphate was contained in the "$KClO_4$-fraction" (Procedure V). These findings indicate that there are at least two types of acid-insoluble labile phosphates in spinach leaves: one, soluble in cold (weak) alkali of pH 8–10, and the other, insoluble at pH 8–10 in the cold but soluble in 2 *N* KOH at 37°C. In repeated experiments the latter form was always obtained in considerably larger amounts than the former.

The behaviors of the two forms of labile phosphorous compounds toward alkali are very similar to those of polyphosphates "B" and "C", respectively, which have been found in *Chlorella* cells (*6*). However, the 2 *N* KOH-soluble labile compound in spinach leaves differs from polyphosphate "C" of *Chlorella* in that it does not precipitate together with $KClO_4$ on neutralization of the KOH-extract with PCA (Procedure V),

TABLE I

Labile Phosphates in Acid Insoluble Fractions of Spinach Leaves

Material		Amount of P[1]			
		*i*P	Δ15P	Δ30P	Total P[2]
"Supernatant"	"pH 9-Extract" (Supernatant in Procedure IV)	0	101	194	2,090
	"2 *N* KOH-Extract" (Supernatant in Procedure V)	4	779	950	1,360
"Precipitate"	"pH 9-Extract" (Supernatant in Procedure IV)	3	145	189	890
	"2 *N* KOH-Extract" (Supernatant in Procedure V)	12	398	446	1,160

1) Amount of P is expressed in μg. present in each fraction obtained from 100 g. of spinach leaves.
2) Determined after digestion with H_2SO_4.

whereas the latter coprecipitates quantitatively with $KClO_4$ under the same condition.

Metachromatic Reaction—When a drop of the clear solution separated from the nucleic acid-fraction by the Ba-precipitation was added to 5 ml. of toluidine blue solution (6 mg. toluidine blue per 1,000 ml. distilled water), a distinct purple colour appeared. This change of colour was not observed on addition of the control solution prepared from the Ba-precipitate of commercial pyrophosphate, thus excluding the possibility that the colour change was due to the pyrophosphate which had been added to the nucleic acid fractions to facilitate Ba-precipitation.

Spectrophotometric observation revealed that the addition of the clear solution obtained from the nucleic acid-fraction to toluidine blue lowers the absorption maximum at 630 mμ and causes an appreciable increase in absorbance in the region of shorter wavelengths. However, a new absorption peak at 530 mμ, as reported by Wiame (*14*) in the case of hexametaphosphate, was not observed in the present experiment. The ratio of E-values at 530 mμ and 630 mμ, which was employed by Wiame as a measure of metachromasy, is shown in Table II, from which it will be seen that a certain substance(s) causing metachromasy is contained in the nucleic acid-fractions of spinach leaves.

Paperchromatography—With the clear solution

TABLE II

Metachromatic Reaction

(Toluidine blue, 6 mg. in 1,000 ml. H_2O)

	$E_{530\,m\mu}/E_{630\,m\mu}$
Water[1]	0.14
Eluate from Ba-precipitate of pyrophosphate[1]	0.15
Eluate from Ba-precipitate of RNA-fraction[1]	0.34
Eluate from Ba-precipitate of DNA-fraction	0.23

1) Two drops of each solution were added to 5 ml. of toluidine blue solution. For further explanation, see text.

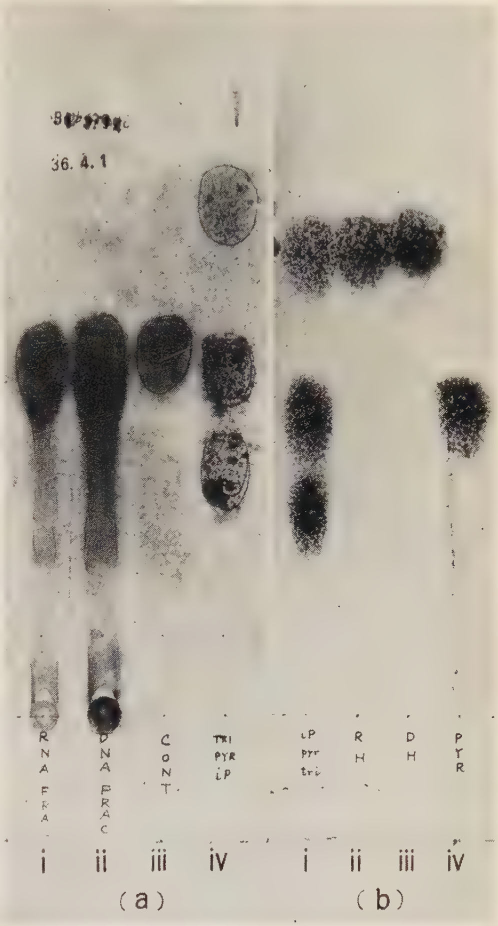

FIG. 1. Paperchromatograms of acid-insoluble labile phosphate.

Solvent used; isopropanol 70 ml., water 30 ml., TCA 4 g., ammonia (20%) 0.4 ml. (*15*). The chromatogram (ascending) was developed for *ca* 20 hours. After spraying of acid molybdate solution, followed by heating at 80°C for 10 minutes, the paper was exposed to ultraviolet radiation, and the phosphate compounds were located by blue spots.

(a) (i) extracts prepared from Ba-precipitate of RNA-fraction; (ii) extracts prepared from Ba-precipitate of DNA-fraction; (iii) control extracts prepared from Ba-precipitate of commercial pyrophosphate; (iv) authentic tripolyphosphate, pyrophosphate and inorganic phosphate.

(b) (i) authentic tripolyphosphate, pyrophosphate and inorganic phosphate; (ii) product obtained by boiling the extract prepared from Ba-precipitate of RNA-fraction (7 minutes in *N* HCl);

(iii) product obtained by boiling the extract prepared from Ba-precipitate of DNA-fraction (7 minutes in *N* HCl); (iv) control extracts prepared from Ba-precipitate of commercial pyrophosphate.

separated from the nucleic acid fractions by Ba-precipitation, one-dimentional paperchromatography was performed using an acid solventa (isopropanol-water-TCA-ammonia) described by Ebel (*15*). The filter paper used was "Toyo" No. 50 or 51, which had been washed in aqueous ethylenediamine tetraacetic acid.

From the results shown in Fig. 1 (a), it may be seen that the phosphate compounds obtained from both RNA- and DNA-fractions did not move from the starting point. After being boiled for 7 minutes in the presence of *N* HCl, the phosphate compound in question (together with pyrophosphate which had been added as carrier) is completely hydrolyzed to orthophosphate (see Fig. 1 (b)), indicating the labile nature of the compound.

It has been shown by Ebel (*15*) and Thilo (*16*) that the mobility of the polyphosphates in the paperchromatogram is quantitatively related to the chain-length of the molecule, and that highly condensed polyphosphates stay at the starting line. Thus, the present results indicate the presence of a highly condensed polyphosphate(s) in the acid-insoluble fraction of spinach leaves.

DISCUSSION AND CONCLUSION

Experimental evidence described above showed that at least two types of polyphosphates are contained in the acid-insoluble fraction of spinach leaves: one, soluble in cold alkali of pH 8–10, and the other, insoluble at pH 8–10 but soluble in 2 *N* KOH at 37°C. It was further shown that in the nucleic acid (RNA and DNA) fractions obtained by Ogur and Rosens' method a labile phosphorous compound (s) is contained, which can be separated from the nucleic acids by Ba-precipitation. The labile compound (s) thus separated gives a positive metachromatic reaction with toluidine blue, and shows no mobility on the paperchromatogram. According to Wiame (*14*) and Ebel (*15*) these features are characteristic of long-chained polyphosphates, while it is not the case with ring-forming metaphosphates. It may, therefore, be concluded that spinach leaves contain at least two kinds of polyphosphates, both or either one of which is a long-chained compound and appears in the acid-insoluble fractions together with RNA and DNA.

SUMMARY

Labile phosphorous compounds were separated from the acid-insoluble fraction of spinach leaves. The nucleic acid fractions obtained from the leaves by the method of Ogur and Rosen also contain a labile phosphorous compound(s) which can be separated from the nucleic acids by Ba-precipitation. The labile compounds thus separated gives a positive metachromatic reaction with toluidine blue, and shows no mobility on the paperchromatogram, indicating that the compound (s) is a long-chained polyphosphates.

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NOTES

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Preparation of *p*-Nitrophenyl S^{35}-Sulfate

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p-Nitrophenyl S^{35}-sulfate (*p*-NPS^{35}) is shown to be a useful substrate in various enzyme reactions. Several methods for the synthesis of *p*-NPS^{35} have been reported (*1–3*), however the following simplified modification of the methods gave the best results so far examined.

EXPERIMENTAL AND RESULT

For the preparation of *p*-NPS^{35}, $H_2S^{35}O_4$ was first converted to S^{35}-labeled chlorosulfonic acid, by the exchange reaction, $S^{35}O_4^{--}+ClSO_3^{-}\rightleftarrows SO_4^{--}+ClS^{35}O_3^{-}$. To a solution of 1–3 mc. of $H_2S^{35}O_4$ (carrier-free) in a small test tube was added the equivalent aqueous NaCl. The aqueous $Na_2S^{35}O_4$ obtained was dried by careful evaporation. 0.08 ml. of dry chlorosulfonic acid was added to the $Na_2S^{35}O_4$ and the test tube stoppered tightly was allowed to stand at 80°C for about 2 hours. After subsequent 24 hours at 37°C, 0.2 ml. of dry chloroform was added to the S^{35}-chlorosulfonic acid solution so formed. The mixture was added dropwise to 60–100 mg. of *p*-nitrophenol in 0.2–0.3 ml. of dry pyridine with stirring and cooling in an ice-salt bath. The mixture was shaken in a tightly stoppered test tube overnight at 30°C. To the reaction mixture 70 mg. of potassium *p*-NPS was added as a carrier. *p*-NPS^{35} was extracted from the reaction mixture with three 1–2 ml. portions of chilled water. Most part of *p*-nitrophenol remained in the residue. The extract was neutralized with $BaCO_3$ and $BaSO_4$ formed was removed by centrifuging. The supernatant was dried by evaporating *in vacuo* carefully in the presence of a small quantity of $BaCO_3$. *p*-NPS^{35} was extracted from the residue with five 3 ml. portions of 80 per cent of hot ethanol. At this stage, most part of $BaCl_2$ was removed as residue. Then ethanol was substituted with water by continuous evaporation and addition of water. To the solution (4 ml.), was added a solution of 2,8-diamino-10-methyl-acridinium chloride (trypaflavin) (*4*, *5*), to a final trypaflavin concentration of 1–2 per cent. The orange yellow precipitate formed of trypaflavin *p*-NPS^{35} was collected by centrifuging and washed with cold water. Then the salt was dissolved in 1 : 2 ethanol-water and to it cation-exchanger (IR-120 K-salt type) was added, with continuous stirring until all the trypaflavin was adsorbed. The colorless filtrate which contained potassium *p*-NPS^{35} was freeze-dried, then faint yellow solid was obtained.

The contaminating inorganic $S^{35}O_4$ in the product was less than 0.2 per cent of that of the products. The yield was 65 mg. The specific radioactivity of the preparation was *ca.* 0.5 μc./μ mole.

If necessary, the product can be further purified as usual by recrystallization.

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Ascorbic Acid Synthesis with the Reconstructed Enzyme System

It has become definitely clear that L-ascorbic acid is formed from L-gulonolactone by a microsomal enzyme in animal tissues (*1*, *2*). The precursor of L-gulonolactone is D-glucuronic acid, and the possibility of the participation of TPN-L-hexonate dehydrogenase and lactonase in the formation of L-gulonolactone from D-glucuronic acid was suggested (*3–4*). In this work, the enzyme system consisting of TPN-L-hexonate dehydrogenase, lactonase and microsomal L-gulonolactose dehydrogenase was reconstructed and the actual formation of L-ascorbic acid from D-glucuronic acid was observed *in vitro*. There are two possible pathways in this case, *i. e.* D-glucuronic acid⟶D-glucuronolactone⟶L-gulonolactone⟶L-ascorbic acid and D-glucuronic acid⟶L-gulonic acid⟶L-gulonolactone⟶L-ascorbic acid. The discussion was made which of these two is the more probable pathway.

PREPARATION AND ASSAY

TPN-L-hexonate dehydrogedase (*5*) and lactonase-I (*3*) were prepared and purified from rat liver according to the previous papers from our laboratory. Microsomes were prepared also from rat liver by the method of Hodgeboom and Schneider and used as L-gulonolactone dehydrogenase (*2*). L-Ascorbic acid formed was determined by the method of Roe and Kuether (*6*).

TABLE I

Ascorbic Acid Formation from L-Gulonic Acid

Enzyme system	Substrate	Ascorbic acid formed (μmole)
Gulonolactone dehydrogenase (Microsomes)	L-gulonolactone (10 μmole)	3.5
	L-gulonic acid (60 μmole)	0
Gulonolactone dehydrogenase (Microsomes) Lactonase-I	L-gulonolactone (10 μmole)	2.6
	L-gulonic acid (60 μmole)	1.8

Reaction mixture contamed 2.0 ml. of 0.2 *M* phosphate buffer (pH 7.0), 0.1 ml. of 0.1 *M* $MgCl_2$, 0.2 ml. of 0.01 *M* reduced glutathione, substrate solution, microsomes prepared from 0.5 g. rat liver, and 40 units of Lactonase-I; total volume, 4.5 ml.; gas phase, oxygen; temperature, 37°C; incubation, 60 minutes.

TABLE II

Ascorbic Acid Formation from D-Glucuronic Acid

Enzyme system	Substrate	Ascorbic acid formed (μmole)
Gulonolactone dehydrogenase (Microsome)	D-glucuronolactone (40 μmole)	0.9
TPN-L-hexonate dehydrogenase	D-glucuronic acid (40 μmole)	0
Gulonolactone dehydrogenase (Microsome) TPN-L-hexonate dehydrogenase Lactonase-I	D-glucuronic acid (40 μmole)	0.4

Reaction mixture contained 0.5 ml. of 0.2 *M* glycylglycine buffer (pH 7.3), 0.2 ml. of 0.02 *M* $MnCl_2$, 0.5 ml. of 0.5 *M* nicotinamide, 0.5 ml. of 0.1 *M* D, L-isocitric acid, 5 mg. of triphosphopyridine nucleotide, 300 units of isocitric dehydrogenase, microsomes prepared from 0.5 g. rat liver, 1,300 units of TPN-L-hexonate dehydrogenase, and 40 units of lactonase-I; total volume, 4.5 ml.; gas phase, oxygen; incubation, 60 minutes; temperature, 37°C.

RESULTS AND DISDUSSION

As can be seen in Tables I and II, L-acorbic acid was formed from L-gulonic acid in the presence of lactonase-I and L-gulonolactone dehydrogenase, and from D-glucuronic acid in the presence of the above two enzymes and TPN-L-hexonate dehydrogenase. When lactonase-I was omitted from these reconstructed systems, the formation of L-ascorbic acid was not obserbed. Previously, it was shown by Yamada (*7*) that in the presence of trapping agent (*e. g.* NH_2OH) D-glucuronic acid could be lactonized non-enzymatically to D-glucuronolactone, whereas L-gulonic acid could be lactonized only enzymatically to L-glonolactone. But in both cases the lactonization did not occur without trapping agent. As lactonase-I was found necessary for the formation of L-ascorbic acid in the above experiments as shown in Table I and II, it seems reasonable to conclude that lactonization occurs at the step of L-gulonic acid in these enzyme systems.

On the other hand, the system consisting of TPN-L-hexonate dehydrogenase and microsomes could synthesize ascorbic acid from D-glucuronolactone, but not from D-glucuronic acid as mentioned above, although the microsomes contained the activity of glucuronolactonase (lactonase-II) (*3*, *8*, *9*). Glucuronolactonase (lactonase-II) seems not to be able to lactonize D-glucronic acid under these conditions.

Kawada *et al.* (*9*) reported that lactonase II could lactonize D-glucuronic acid to D-glucuronolactone only in the presence of trapping agent as same as lactonase I.

Further, the system of lactonase-I plus microsomes synthesized a substance from L-xylonic acid, which is produced by bio-degradation of ascorbic acid, with 2, 3-diketo aldonic acid decarboxylase (*10*). This substance could be assayed by the method of Roe and Kuether and was supposed to be L-erythroascorbic acid.

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